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UNIVERSITY OF ALBERTA

KIDNEY TUBULAR CELL SHEDDING IN EXPERIMENTAL ACUTE RENAL FAILURE

By

Yu-Ling Li

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN
PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

IN

EXPERIMENTAL PATHOLOGY

DEPARTMENT OF PATHOLOGY

EDMONTON, ALBERTA

FALL, 1991

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UNIVERSITY OF ALBERTA
FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled **KIDNEY TUBULAR CELL SHEDDING IN EXPERIMENTAL ACUTE RENAL FAILURE** submitted by **YU-LING LI** in partial fulfilment of the requirements for the degree of **MASTER OF SCIENCE** in **EXPERIMENTAL PATHOLOGY.**

Date: Sept. 30, 1991

To my Parents

ABSTRACT

In the search for the pathogenesis of acute renal failure (ARF), four principal mechanisms have been proposed: renal tubular obstruction, renal tubular backleak, renal vasoconstriction, and decreased renal glomerular ultrafiltration coefficient. Recently, experimental data of renal tubular cell shedding during ARF have been accumulating. This renal tubular cell shedding could represent renal tubular cell exfoliation in situ and the latter could cause renal dysfunction through tubular obstruction, tubular backleak, and renal vasoconstriction. This information prompted the formation of the hypothesis that renal tubular cell exfoliation may play an important role in ARF. This study was designed to test this hypothesis by systematically investigating the renal tubular cell shedding phenomenon in early phases of rabbit models of ischemic and toxic ARF.

Studies of renal tubular cell shedding in the early phase of rabbit models of ARF induced by renal artery clamping, glycerol, and mercuric chloride were performed using light microscopy, trypan blue viability test, and flow cytometry.

Increased number of renal tubular cells were found in urinary sediments in all the experimental animal groups immediately after the insult was induced and persisted for the whole 6-h experimental period. Large number of viable tubular cells were present in the voided urine in all experimental groups. The per cent of viable tubular cells was characteristic of the individual model. Proximal tubular cells comprised the majority of the voided tubular cells as analyzed by flow cytometry. Total cell and total live cell shedding were correlated with urine output suggesting a dependence of cell shedding on urine flow. Viability of voided tubular cells was correlated with renal function indicating degree of cell damage was associated with renal function. Total cell and concentration of voided viable cell were correlated with renal function suggesting that cell shedding may reflect degree of tubular obstruction.

The study demonstrated an association between renal tubular cell shedding and renal dysfunction in ARF in early phases of rabbit models of ARF, providing the experimental evidence for the hypothesis that renal tubular cell exfoliation is an important factor in the pathogeneses of ARF.

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LIST OF ABBREVIATIONS

ARF	Acute Renal Failure
ATN	Acute Tubular Necrosis
GFR	Glomerular Filtration Rate
FC	Flow Cytometry
FDA	Fluorescein Diacetate
AP	Alkaline Phosphatase
GGT	Gamma-Glutamyl-Transpeptidase
SUN	Serum Urea Nitrogen
SCR	Serum Creatinine
C_{UN}	Urea Nitrogen Clearance
C_{CR}	Creatinine Clearance
LM	Light Microscopy
FALS	Forward Angle Light Scatter
90LS	90° Light Scatter
PTC	Proximal Tubular Cell
RBF	Renal Blood Flow

LIST OF ABBREVIATIONS

ARF	Acute Renal Failure
ATN	Acute Tubular Necrosis
GFR	Glomerular Filtration Rate
FC	Flow Cytometry
FDA	Fluorescein Diacetate
AP	Alkaline Phosphatase
GGT	Gamma-Glutamyl-Transpeptidase
SUN	Serum Urea Nitrogen
SCR	Serum Creatinine
C_{UN}	Urea Nitrogen Clearance
C_{CR}	Creatinine Clearance
LM	Light Microscopy
FALS	Forward Angle Light Scatter
90LS	90° Light Scatter
PTC	Proximal Tubular Cell
RBF	Renal Blood Flow

CHAPTER ONE

INTRODUCTION

1.1. THE PATHOGENETIC DEBATE CONCERNING ACUTE RENAL FAILURE

Acute renal failure (ARF) is a clinical syndrome characterized by a rapid decrease in renal function. Its clinical hallmark is rapidly progressive azotemia which is usually but not always accompanied by oliguria. Clinically, the causes of acute renal failure are many and have been traditionally divided into prerenal, renal, and postrenal. Thus, acute renal failure includes clinical states associated with volume depletion and obstructive uropathy as well as primary renal abnormalities. When acute renal failure is of renal origin, its cause can be ascribed to such forms of renal injury as acute and rapidly progressive glomerular nephritis, acute interstitial nephritis, cortical necrosis, intrarenal obstruction, renal arterial or venous obstruction, and an entity called acute tubular necrosis (ATN) which is the destruction of renal tubular epithelial cells caused either by direct poisoning of the tubules of various noxious agents, such as heavy metals, or by renal ischemia. The present study is only concerned with this last subclass of ARF.

Extensive research has been performed on elucidating the mechanism of renal insufficiency in ARF both in clinical settings and in a variety of animal models. However, a unifying theory to account for the pathogenesis of ARF is still out of reach. The exact mechanism of the defect in glomerular filtration rate (GFR) in acute renal failure seems to be multifactorial and varies from one animal model to another. A factor judged important in causing ARF in one study often seemed less important in another. It is not clear whether reported differences in pathogenesis reflect fundamental differences between different models, differences in the techniques employed, or the fact that different stages in the same process were being evaluated.

Four principal mechanisms of renal insufficiency in ARF have been proposed (Levinsky et al., 1981; Barrym et al., 1986; Michel et al., 1987; Solez, 1984; Stein et al., 1978), two tubular; tubular obstruction, tubular leakage, and two vascular; persistent vasoconstriction/decreased renal blood flow, and a decrease in the glomerular ultrafiltration coefficient (Fig. 1).

1.1.A. TUBULAR OBSTRUCTION

During ARF, renal tubules may be obstructed by elements such as cellular debris, casts, or interstitial edema. This obstruction could allow an increase in ultratubular pressure sufficient in magnitude to offset effective glomerular filtration pressure, thus causing decreased glomerular filtration rate and renal insufficiency (Flamenbaum, 1984).

Evidence for tubular obstruction has come from

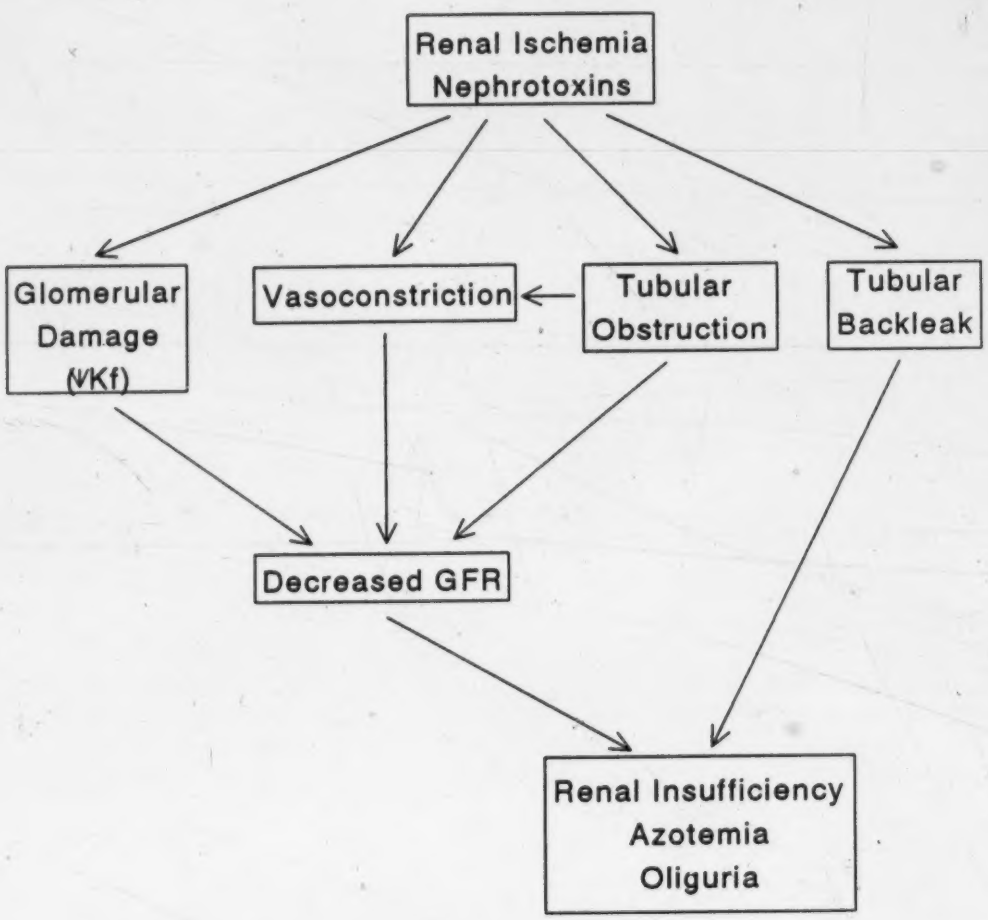

FIGURE 1. Schema of pathogenesis of renal insufficiency in ARF.

morphological and functional studies. On histologic sections the strait portion of proximal tubules are occluded by swollen blebs of microvilli desquamated from the brush border of the proximal tubule pars convoluta (Donohoe et al., 1976). Casts are often seen in the more distal part of the nephron. Microdissection studies also confirmed the existence of long branching casts obviously capable of obstructing the tubules (Oliver et al., 1951). It has been suggested that these casts are formed from break-down products of the cellular debris which combine with Tamm-Horsfal protein produced in the ascending limb of Henle (Rutechi et al., 1971). This protein has the physicochemical properties that tend to make it precipitate in tubules under the conditions of urine flow and composition likely to be present during ARF (Patel et al., 1964).

In functional studies of experimental ARF, direct measurement of intratubular pressure can be made, which demonstrates an increased intratubular hydrostatic pressure (Finn, 1979; Donohoe et al., 1978). This probably is the most convincing evidence for tubular obstruction.

Tubular obstruction has been established in ARF associated with arterial occlusion. However, evidence is inconclusive in ARF caused by glycerol and mercuric chloride (Levinsky et al., 1981). For human ARF, evidence both for and against this mechanism exists (Solez, 1984).

1.1.B. TUBULAR BACKLEAK

Tubular epithelial cell damage during ARF may allow the transtubular leakage of a normally formed glomerular filtrate to return to the systemic circulation through renal lymphatics. Azotemia may thus result from this abnormal tubular permeability.

Evidence supporting this backleak theory of pathogenesis of ARF has derived from two lines of investigation. Histologically, bare areas are often seen along the tubular basement membrane where individual tubular cells are missing. This cell loss is significantly less prominent after functional recovery although most of the other lesions persist into the recovery period in human ARF, suggesting that this cell loss is functionally important (Solez et al., 1979). Tubulorrhexis, a type of tubular lesion that destroys both the epithelial cells and the underlying basement membrane often exists in ischemic ARF. Occasionally there are large masses of extravasated tubular fluid in interstitium and interstitial edema may also be present. More directly tubular leakiness has been demonstrated by backleak of marker molecules such as inulin (Tanner et al., 1973; Blantz, 1975; Burre et al., 1977; Hanley, 1982), lissamine green (Arendshorst et al., 1975), and horseradish peroxidase (Donohoe, 1978) injected intravenously or intratubularly. The fact that intravenously injected markers penetrated tubule epithelial cells in post-ischemic but not in normal rat kidney argues strongly that backleak is

a real phenomenon contrary to the opinion held previously by some investigators that backleak may be an artifact caused by excessive tubular pressure during intratubular microinjection of markers.

Evidence against this mechanism comes from the lack of quantitative correlation between the morphological changes and the renal function. Thus, one can observe marked diminutions in renal function in either experimental or clinical ARF without definite histologic changes and vice versa. However, this lack of correlation does not exclude the possible role played by backleak in causing ARF if one can explain the paradox in the light of the following considerations: 1) the severity of the disease appears to correlate more with the number of the nephrons damaged than with the amount of renal mass affected; 2) small breaks in nephron integrity or continuity may be adequate to cause considerable loss of function; 3) the sampling error or superimposition of post mortem autolytic changes in tissues obtained at necropsy cause small foci of necrosis to go unnoticed; 4) the disease varies with time during the course of the illness.

Backleak of markers has been demonstrated in the renal artery occlusion and mercuric chloride models. No backleak has been observed in the glycerol model (Levinsky et al., 1981). In human ARF this mechanism is supported by histologic lesions and Dextran fractional clearance data (Myers et al., 1980).

Tubular backleak and tubular obstruction are interrelated as pointed out by Solez (1984). The increased intratubular pressure brought about by obstruction may increase backleak. Conversely if extensive backleak occurs, intratubular pressure can not rise to a level that will expel obstructive tubular casts.

1.1.C. DECREASED RENAL BLOOD FLOW

Renal blood flow (RBF) has been demonstrated to be reduced both in patients with ARF and in the early phases of all principal animal models after arterial occlusion, norepinephrine, glycerol, mercury etc.(Gross et al., 1982; Hollenberg et al., 1968; Hollenber et al., 1970; Levinsky et al., 1981; Reubi et al., 1973). However, in the arterial occlusion, glycerol, mercury, and uranyl nitrate models, RBF spontaneously increases to normal within the first 24 to 48 hours after the initiating renal injury, suggesting that although decreased RBF is clearly an initial pathophysiological feature of both ischemic and nephrotoxic ARF, it is equally evident that it is neither necessary nor sufficient to the maintenance of renal failure (Levinsky et al., 1981).

Many observations have suggested an important role for active renal vasoconstriction as the factor responsible for the reduction in renal blood flow in ARF. Direct physiological and pathological studies suggest that the increased renal resistance during ARF is due to vasoconstriction of

arterioles, the primary renal resistance vessels (Arendshorst et al., 1975; Daugharty et al., 1974; Venkatachalam et al., 1976). In all these studies resistance was increased in both the afferent and the efferent arterioles, contrary to the hypothesis that the low GFR in ARF may be due to afferent vasoconstriction coupled with efferent vasodilation (Reubi, 1974). Renal arteriograms in patients with ARF also showed constriction of arcuate and segmental arteries as well as vasoconstriction of the arterioles (Hollenberg and Adams, 1974).

Renin-angiotensin system has been singled out as the most likely mediator responsible for the renal vasoconstriction in ARF. Since its first proposition by Goormaghtigh based on altered morphology of the juxtaglomerular apparatus in human ARF (Goormaghtigh, 1945), investigations relevant to the role of the renin-angiotensin system in the pathogenesis of ARF have been performed along several lines: measurements of plasma and intrarenal renin and angiotensin during various times in the course of the syndrome; attempts to reproduce the renal lesion by administration of angiotensin; modification of the course by maneuvers designed to suppress the renin-angiotensin system, especially with a supplemented sodium intake; and attempts to blunt the action of angiotensin with antibodies or with pharmacologic agents designed to block the system at one of several levels. Despite the enormous effort by many investigators it is still not possible to provide a conclusive statement on renin's role in the pathogenesis of ARF (Hollenberg, 1983). Evidence both for and against its role exists.

Activation of the renin-angiotensin system during ARF has been proposed to be caused by a tubuloglomerular feedback mechanism by Thurau and associates (Mason et al., 1978; Mason, 1976; Thurau and Boylan, 1976). According to this hypothesis damage to the reabsorptive capacity of the proximal convoluted tubule or the loop of Henle increases salt concentration at the macula densa, thereby activating the tubuloglomerular feedback mechanism to decrease blood flow and filtration in the injured nephron. Increased distal tubule sodium concentration and renin activity in individual juxtaglomerular apparatus constitute the evidence for this mechanism (Flamenbaum et al., 1976; Flamenbau et al., 1976a; Thurau et al., 1976).

Other causes of reduced renal blood flow have also been investigated. Arendshorst, Finn, and Gottschalk (Arendshorst et al., 1975; Finn et al., 1975) have suggested that tubular obstruction may cause reduced RBF. They found that 24 hours after the tubules were obstructed by microinjection of viscous oil, blood flow to the corresponding glomeruli was reduced while the neighbouring nonobstructed nephrons had normal blood flow. Some variant of the tubuloglomerular feedback mechanism is probably responsible for this link between obstruction and arteriolar constriction. However, during recovery from

postischemic ARF, RBF remains severely reduced long after intratubular obstruction has improved (Finn and Chevalier, 1979). Thus, the connection between obstruction and persistent ischemia is uncertain.

Another hypothesis for decreased RBF in ARF is that the increased vascular resistance is due to deficient synthesis of a renal vasodilator such as one of the prostaglandins (Fine, 1970; Held, 1976) or kallikrein (Martin et al., 1976). Inhibition of renal prostaglandin synthesis by indomethacin potentiates the induction of ARF by glycerol (Torres et al., 1975). Renal medullary tissue explanted to the abdominal wall mitigates the severity of ARF after arterial occlusion possibly by secreting prostaglandins (Held, 1976). On the other hand, infusion of a prostaglandin is not protective against ARF caused by glycerol or uranyl nitrate (Goffi et al., 1975) and blockade of prostaglandin synthesis does not increase the severity of ARF after administration of mercury (Torres et al., 1975). Thus, information is inadequate to decide whether prostaglandin deficiency is a pathophysiological mechanism in ARF. Information on the possible role of the renal kallikrein-kinin system in ARF is limited to the observation by Martin and associates that urinary kallikrein excretion is greatly reduced in methaemoglobin-induced ARF in rats (Martin et al., 1976).

Renal hemodynamics do not, however, correlate well with alterations in renal function in some circumstances. Vasodilators or volume expansion may return renal blood flow to normal values without improving renal function (Mauk et al., 1977; Leaf, 1970). After an initial decrease, renal blood flow may return to normal even though glomerular filtration rate remains depressed (Ladefoged and Winkler, 1971; Sudo et al., 1977). Significant abnormalities in renal blood flow may not be demonstrable during the course of ARF. Neither immunization against angiotensin II nor administration of specific antagonists of the renin-angiotensin system could be shown to have a beneficial effect on renal function (Ishikawa and Hollenberg, 1976; Oken et al., 1975; Mason et al., 1979). It may be that the renal vasculature in ARF cannot autoregulate blood flow when systematic pressure is reduced, making the kidney susceptible to further ischemic injury.

Vasoconstriction observed in the larger intrarenal vessels in ARF can not be angiotensin induced but could be caused by catecholamines or vasopressin. Three agents that block the kidney's response to vasopressin; clonidine, lithium, and demeclocycline lesson the severity of renal failure in a pedicle-clamping model of ARF in rabbit suggesting a role played by vasopressin in renal insufficiency in this model (Solez, 1984).

1.1.D. DECREASED GLOMERULAR ULTRAFILTRATION COEFFICIENT (Kf)

A fall in glomerular ultrafiltration coefficient has only been directly demonstrated by micropuncture study in the

initial phase of ARF due to uranyl nitrate (Blantz, 1975) and in renal failure due to gentamicin (Baylis et al., 1977). Indirect evidence for this phenomenon has been noted in the norepinephrin (Cox et al., 1974) and mercuric chloride (Baehler et al., 1977) models.

Abnormal glomerular morphology has been described during certain phases of ARF in several models (Flamenbaum et al., 1976; Dach and Kurtzman, 1976; Cox et al., 1974; Avasthi et al., 1979; Avasthi et al., 1980; Stein et al., 1975). These changes include decrease in the number and total area of glomerular capillary endothelial fenestrae (Avasthi et al., 1980; Blantz and Konnen, 1975), abnormal epithelial cell processes and glomerular deposits (Dach and Kurtzman, 1976; Venkatachalam et al., 1976).

There is thus far no direct evidence for decreased Kf in human cases of ARF. Indirect evidence includes flattening of glomerular podocytes observed early after an ischemic insult in transplanted human kidneys and the eventual severity of postischemic acute renal failure suggests that these podocyte changes are functionally important (Solez, 1981).

The mechanisms of decreased Kf are uncertain. It may include decreased glomerular capillary permeability and reduced capillary surface area.

1.2. KIDNEY TUBULAR CELL SHEDDING PHENOMENAN AND THE ROLE OF TUBULAR CELL EXFOLIATION IN THE PATHOGENESIS OF ARF

Renal tubular cells have been found in urine in clinical ARF. Urinary sediment from patients with ARF has been typically described as the presence of muddy-brown casts, course granular casts, red blood cell casts, red blood cells, white blood cells, and renal tubular cells. The phenomenon of tubular cell shedding during ARF has regained attention recently perhaps because of two reasons. First, the development of new techniques such as electron microscopy and monoclonal antibody staining allows more detailed studies of the characteristics of the voided cells. Secondly, the cell loss phenomenon along tubular epithelium correlates with renal function in clinical ARF (Solez, 1979) suggesting that this cell loss and the closely related cell exfoliation may contribute importantly to renal dysfunction in ARF.

Cell shedding in ARF has been studied by several investigators using different techniques. Lindqvist et al. (1975) studied urine sediments from patients with various renal or urinary tract diseases by phase contrast microscopy. They defined renal epithelial cells in urine as cells with rounded, eccentrically located, light-grey nuclei in which the nucleoli were visible. These renal epithelial cells were slightly larger than or sometimes twice as large as polynuclear leucocytes. Polynuclear granulocytes were described as cells with two or more dark, coupled nuclei and bright cytoplasm with highly motile granules. Mononuclear leucocytes were classified as cells of the same size as the

polynuclear granulocytes, with round or kidney-shaped, dark nuclei, and a light granular cytoplasm. They also found that urine specimens of patients with ARF had on average 60.5% polynuclear granulocytes, 18% mononuclear leucocytes and 36.5% renal epithelial cells.

A profile of cells in urine obtained from patients with ATN has been provided by the study of Segasothy et al (Segasothy et al., 1988; Segasothy et al., 1989) using monoclonal antibodies as cell identification markers. The study revealed that proximal tubular cells comprised the majority of the shed cells in urine (36.5%). Cells from the loop of Henle, distal tubule, and collecting duct combined were slightly less numerous than proximal tubular cells (30%). 15% granulocytes were found and few monocytes, glomerular epithelial cells , and urothelial cells were also present (3%, 3%, and 9% respectively).

Mandal et al. (1985) have studied urinary sediment in human ARF using electron microscopy. By this technique renal tubular cells were recognized as cells with abundant cytoplasmic organelles, particularly mitochondria. Proximal tubular cells were qualified as cells with microvilli. The study revealed that the major cell population observed in urine sediments from patients with ARF was of renal tubular origin, and the severity of ultrastructural alterations seen in urinary renal tubular cells correlated with the severity of the clinical course, renal recovery and patient survival.

Tubular cells shed in urine have also been found to be alive and culturable (Racusen et al., 1988).

Based on the above observations, it is appropriate to postulate that renal tubular cells detach from the renal tubular basement membrane during ARF, which in turn may cause renal dysfunction presumably by causing directly tubular obstruction and tubular backleak, and indirectly renal vasoconstriction (Fig. 2).

1.3. INTRODUCTION TO FLOW CYTOMETRY

Flow cytometry (FC) is a process in which measurement of physical and/or chemical characteristics of cells or biological particles are made while the cells or particles pass in single file, through the measuring apparatus in a fluid stream.

1.3.A. COMPONENTS OF A FLOW CYTOMETER

Flow cytometers are usually composed of four basic systems (Willman and Stewart, 1989; Lovette III., 1984); 1) a light source, usually a laser, 2) a sample chamber, 3) an optical system that directs scattered or emitted light from the cell to an electric system that converts light impulses (photons) to discrete digital signals and, 4) a computer system that receives the digital pulses and performs data analysis as well as controls the operation of the instrument.

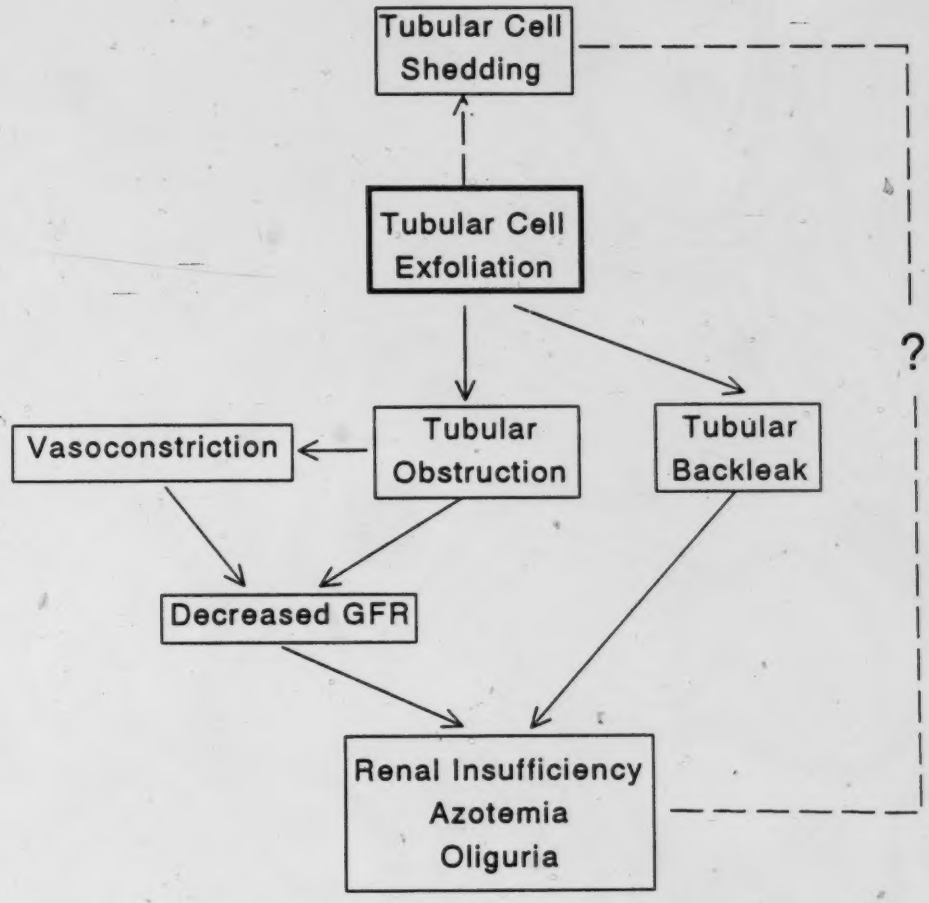

FIGURE 2. Possible role of renal tubular cell exfoliation in pathogenesis of ARF and the relationship between urinary renal tubular cell shedding and renal function

1.3.B. GENERAL PRINCIPLES OF FLOW CYTOMETRY

Within the flow cytometer sample chamber, a sheath flow is forced under pressure into a V-shaped nozzle assembly and exits through a small orifice. The geometry of the nozzle creates a hydrodynamic focusing effect and draws the sample fluid into the stream and surrounds it with sheath fluid. The cells within the sample fluid must then pass single file through the pin point light source after exiting the nozzle assembly, much like beads on a string (Fig. 3).

As cells in the sample stream pass through the nozzle in single file and bisect the laser light source, three types of simultaneous measurements may be made (Fig. 3); 1) light scattered in the forward direction (Forward Angle Light Scatter-FALS) roughly correlates with cell size, 2) light scattered 90 degrees from the light source (90 degree Light Scatter-90LS) reflects cytoplasmic granularity, and 3) fluorescence emitted from various light-activated fluorochromes bond to the cells under analysis is also detected 90 degrees from the light source. All these signals can be measured simultaneously for each cell allowing correlations among various parameters.

The photons of light falling upon the detectors are converted by photomultiplier tubes to an electrical impulse (an analog signal). This signal is processed by an analog-to-digital converter which converts the analog impulse to a digital signal: a number. This number is recorded by the computer system as a data point in a frequency distribution or histogram, with up to 1024 channel resolution.

1.3.C. DATA PRESENTATION AND ANALYSIS OF FLOW CYTOMETRY

1.3.C.a. UNIVARIATE DATA ANALYSIS

For univariate data analysis, the data are plotted as a histogram (frequency distribution); number of counts per channel versus the channel number (Fig. 4). The amplitude of the measured variable is digitized and becomes represented as the channel number; the greater the magnitude of the variable, the higher the channel number. Thus the abscissa (X) of the plot represents the magnitude of the variable. The analyzers have a finite number of channels, often 256. Thus, each channel covers a small range of amplitudes. The ordinate (Y) of the plot is the number of objects (e. g. cells or chromosomes) that have an amplitude that falls within the channel width, producing a frequency distribution (Dean, 1990).

1.3.C.b. MULTIVARIATE DATA ANALYSIS

The most common form of data presentation and analysis for two parameter measurements via flow cytometer is a dot plot in which one parameter is plotted against the other (Fig. 5). The intensity at any point of the plot gives some indication of the frequency with which the corresponding

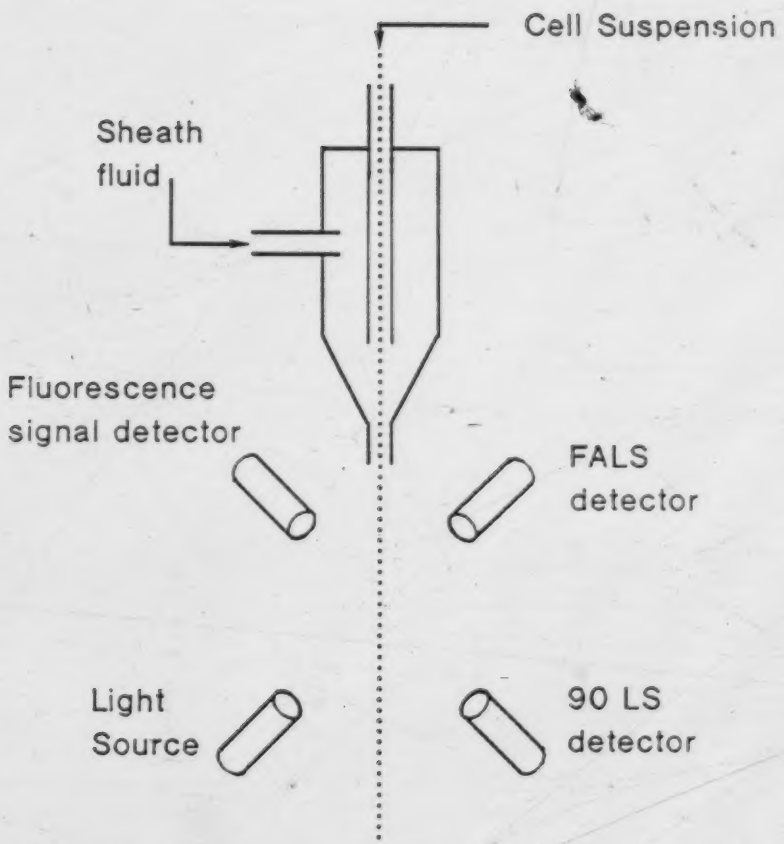

FIGURE 3. Schema of a flow cytometric system.

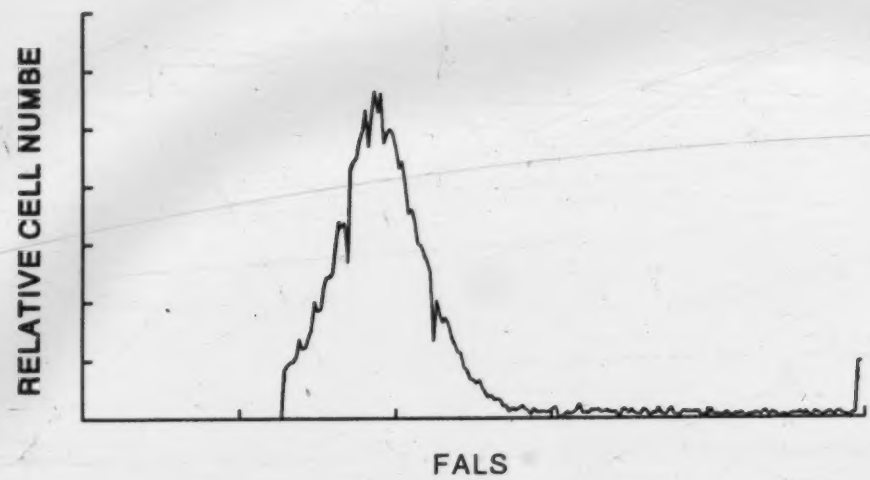

FIGURE 4. One parameter histogram of FALS (horizontal axis) vs number of cells (vertical axis).

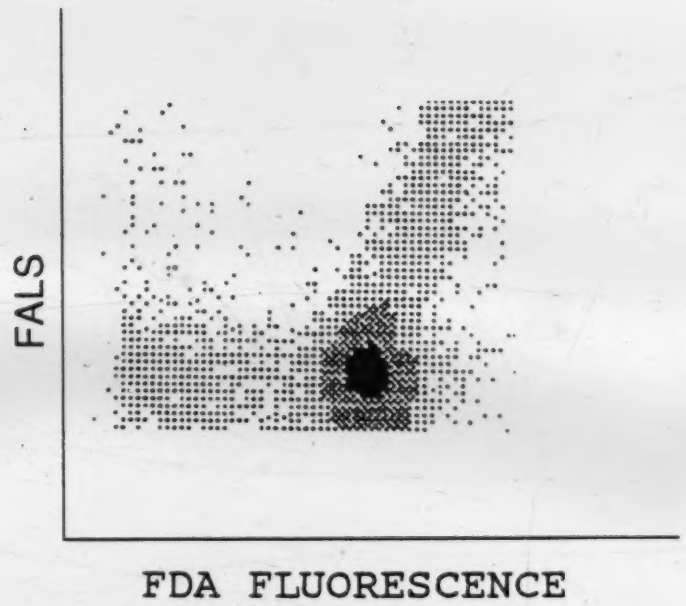

FIGURE 5. Two parameter dot plot of FDA fluorescence (horizontal axis) vs FALS (vertical axis). The third dimension is the number of cells.

values of the variable appear in the sample.

While one parameter histogram sometimes gives poor resolution of a heterogenous cell population, it is almost always possible to distinguish different cell types in the sample by multivariate frequency distributions of two or more parameters.

1.3.D. PARAMETERS MEASURED BY FLOW CYTOMETRY

Depending upon the composition of a cellular sample and upon the experimenter's goals, parameters can be used both to differentiate one cell type from others in a mixed population and to provide information about the cells' biological state. The number of measurable cellular parameters by flow cytometry is very extensive. Based upon Shapiro's classification these parameters can be divided into two groups (Shapiro, 1988); 1) intrinsic parameters which are measured without using reagents include measurements of such cellular properties as cell size, cytoplasmic granularity,and autofluorescence, 2) extrinsic parameters require reagents for their detection by the flow cytometer. They include parameters such as DNA content, surface antigen, and enzyme activity.

The following discussion will describe those parameters used in this study.

1.3.E. FORWARD ANGLE LIGHT SCATTER IS A ROUGH ESTIMATION OF CELL SIZE THAT CAN BE USED TO DISTINGUISH DIFFERENT CELL TYPES

Theoretically, a linear relationship exists between the size and small angle (0.5-2.0 degrees) light scatter of smooth spherical objects with a certain range of diameters and the same refractive index. Using a flow cytometer, Mullaney et al. (1969) demonstrated that the intensity of light scattered at small angles from an incident laser beam was proportional to particle volume. Since then, flow cytometry forward angle light scatter measurements have been widely used for estimation of cell size.

However, many factors other than cell size influence cells' forward light scattering (Shapiro, 1977; Kerker, 1983; Weil and Chused, 1981; Wott et al., 1980; Sharpless et al., 1977; Salzman et al., 1979; Salzman et al., 1979a; Salzman et al., 1975; Loken and Honk, 1981; Loken et al., 1976; Loken and Herzenberg, 1975; Kerker et al., 1979; Salzman et al., 1975; Sharpless, 1976; Sharpless et al., 1975). These factors include instrumental factors such as the wavelength of light used and the precise range of angles over which light is collected. Cellular properties other than cell size also influence forward angle light scattering, e.g., differences in refractive index between the cells and the surrounding medium, cells' internal structure, and the presence within or upon cells of materials with strong absorption at the illumination wavelength used. McGann et al. (1988) demonstrated, using human lymphocytes and hamster fibroblasts, that the intensity

of FALS varied inversely with cell volumes in hypertonic and hypotonic solutions.Therefore, when interpreting results of cells' forward angle light scatter, one must bear in mind that FALS is not an accurate measure of cell size.

In spite of the limitations of FALS, it is still a useful parameter for cell identification and differentiation. Limited discrimination of leucocyte populations can be effected with FALS alone. In a study of P fimbriae receptor accessibility on uroepithelial cells, Jacobson et al. (1986) used FALS as a gating parameter to select the uroepithelial cells from the urine.

1.3.F. MEASUREMENT OF ENZYME ACTIVITY BY FLOW CYTOMETRY PERMITS DISCRIMINATION OF CELLS WITH VARYING LEVELS OF ENZYME ACTIVITY

Rapid and quantitative determination of enzyme activity in single cells can be achieved by flow cytometry. Cells that differ in an enzyme or isoenzyme by more than 3- to 4-fold may be distinguished using this method (Dolbear, 1983). With present instrumentation and staining techniques, enzyme level or activity can be analyzed in three ways: by absorbency staining, with fluorogenic substrates, and by immunofluorescent staining. Use of fluorogenic substrates to measure cellular enzymes increases the sensitivity of the assay 1000-fold over that of colorimetric methods (Dolbear and Smith, 1977).

Fluorogenic substrates are fluorochromes attached with a functional substrate group. When altered upon by specific enzymes they are converted into products that have either increased fluorescence or shifted spectra. Most fluorogenic substrates have been constructed from chromophores that have either an amino or a hydroxyl group that participates in the electronic conjugation. If the product of the enzymatic cleavage of the fluorogenic substrate is too soluble, it can not be retained by the cell, consequently, the fluorescence can not be detected. A trapping agent may be used to capture the liberated fluorescence. Trapping agents are also useful in wavelength shifting and enhancing of the fluorescence.

1.3.G. FLUORESCEIN DIACETATE AS A PROBE FOR CELLULAR NONSPECIFIC ESTERASES

Fluorescein diacetate (FDA) is the ester of fluorescein. It is uncharged and lipophilic and thus readily crosses cell membranes. Once inside the cell, ester molecules are readily hydrolysed to fluorescein and acid residues by cellular nonspecific esterases which are present in almost all cells. The free fluorescein anion is restricted by intact cells and renders the cell highly fluorescent. The fluorescein molecules are lost by cells with damaged membranes (Leaf et al., 1983).

The intracellular accumulation of fluorescein can be monitored quantitatively by using a flow cytometer with the appropriate filters. This measurement has been used both as a

parameter for cell viability and a parameter for cell differentiation.

1.3.G.a. FDA BASED VIABILITY ASSAY

Many techniques have been employed to determine cell viability. These techniques measure those cell functions such as reproductive capability, e.g., clonigenic assay; cells' metabolic state, e.g., stimulation of respiration with exogenous substrate; synthesis of DNA; synthesis of protein; and membrane permeability, e.g., dye exclusion tests.

Perhaps the most frequently used viability test is dye exclusion test. Dye molecules do not permeate cells that are alive and have intact membranes. Cells that are injured take up the dye and can easily be distinguished from normal cells. A number of dyes have been used, including methylene blue (Schrek, 1936), eosin (Hanks and Wallace, 1958), erythrosin B (Philips and Terryberry, 1957), nigrosin B (Kaltenback et al., 1958), and lissamine (Holmberg, 1961). Trypan blue, however, is the dye most commonly used.

The viability of cells has also been monitored by the uptake and hydrolysis of FDA (Rotman, 1966). Staining with fluorescein has been closely correlated with exclusion of trypan blue when both techniques have been compared (Kreisberg, 1980; and Schanne, 1977). However, by using flow cytometer, FDA based viability assay has many advantages over conventional method such as trypan blue exclusion method. First, it is rapid by utilizing flow cytometric methods to quantitate intracellular fluorescence. It is possible to analyze viability of hundreds of thousands of cells rapidly, while trypan blue exclusion is slow and rather laborious as it requires counting the cells under the microscope. Secondly, flow cytometric FDA based viability assay is very reliable. Dead cells only give very low background levels of fluorescence (Schols, 1988), and viable and nonviable cells are found in widely separated regions of the fluorescence histogram, and are easily and accurately quantitated (Bell et al., 1988). There is little or no interobserver variation in the interpretation of the result.

1.3.G.b. FDA STAINING OF NONSPECIFIC ESTERASE ACTIVITY AS A PARAMETER FOR CELL DISCRIMINATION

FDA not only stains live cells but also stains live cells differently. With the aid of flow cytometry, FDA , a widely used viability stain can also be used to rapidly identify and quantitate discrete subpopulations in a heterogeneous sample (Buttke, 1983).

Early in 1966 when Rotman and Papermaster (1966) first discovered the intracellular accumulation of fluorescein fluorescence by viable cells after incubation with FDA, which they termed fluorochromasia, they also found that a heterogeneity of the reaction existed among different types of cells studied. They demonstrated that primary isolates of

mammalian tissues showed wide range in the degree of fluorochromasia, varying between positive and completely negative cells. They found that human and mouse erythrocytes were negative and chicken erythrocytes had positive nuclei only. They attributed the variations of the fluorochromatic reaction to differences in cell membrane which occurred during growth and differentiation or possibly genetic heterogeneity of the cell population.

In an attempt to identify a biochemical marker for distinguishing between B and T lymphocytes, numerous workers have compared the two cell types for their nonspecific esterase activities (Mueller et al., 1975; Kuleukompff et al., 1977; Tofferman et al., 1977). It has been shown that T lymphocytes display more a-naphthyl-acetate esterase activity than B cells. Similarly, it was also found that human (Wilder and Cram, 1977) and mouse (Buttke et al., 1983) T and B lymphocytes interact differently with FDA, resulting in two populations of differing fluorescence. The difference in fluorescence can be resolved by flow cytometry and T cells were found to have higher fluorescence intensity than B cells (Buttke et al., 1983).

Differences in cells' fluorescence accumulation after incubation with FDA probably reflect differences in their intracellular nonspecific esterase activities and differences in their membrane permeabilities for FDA, fluorescein or both.

Cytochemical methods for nonspecific esterase have been used for the identification and differentiation of blood cells. It was found that nonspecific esterase activity was strong in monocytes and weak in granulocytes and lymphocytes and T lymphocytes had higher nonspecific esterase activity than B lymphocytes (Yam et al., 1970) (Table 1).

Nonspecific esterase histochemistry of rabbit kidney showed that proximal convoluted tubules stained most intensively. Distal convoluted tubules and Henle's loops stained slightly and glomeruli and collecting tubules were negative (Malaty and Bourne, 1955).

1.3.H. FLUORESCENT STAINING OF ALKALINE PHOSPHATASE AS A MARKER FOR RENAL PROXIMAL TUBULAR CELL.

A flow cytometric assay for AP has been developed by Dolbear et al. (1980). Naphthol AS-BI phosphate was used as the fluorogenic substrate, and by using the coupling agent fast red TR, the diffusible naphthol AS-BI product was trapped inside the cell making it possible to measure the product fluorescence by flow cytometer. Fast red TR also shifted the fluorescence of the naphthol AS-BI to the near infrared region of the spectra. Using this method they discriminated normal from transformed WI-38 cells.

Alkaline phosphatase has been used as a marker enzyme for renal proximal tubular cell. In kidney, only staining of the brush border of proximal tubules could be demonstrated by histochemical method (Burstone, 1958).

TABLE 1

NONSPECIFIC ESTERASE ACTIVITY OF BLOOD CELLS ASSESSED BY CYTOCHEMICAL METHOD
(Modified from Yam et al., 1970)

Cell Type	Nonspecific Esterase Activity
Monocytes	++++
Histiocytes	++++
Plasma Cells	0-++
Eosinophils	0-+
Lymphocytes	0-+
Neutrophils	0-+
Basophils	0

Blood cells also contain AP activity. By light microscopic cytochemical staining, macrophages and neutrophils are positive. Eosinophils occasionally show some weak cytoplasmic stain in the background. Basophils are normally quite negative. lymphocytes, monocytes, erythrocytes and platelets are generally quite negative, although a very few normal lymphocytes, may show a weak or moderate cytoplasmic reaction.

1.3.I. FLUORESCENT STAINING OF GAMMA-GLUTAMYL-TRANSPEPTIDASE AS A MARKER FOR RENAL PROXIMAL TUBULAR CELL

A fluorometric cytochemical assay for gamma-glutamyl-transpeptidase (GGT) using the substrate gamma-glutamyl-4-methoxy-2-naphthylamide and the trapping agent 5-nitrosalicylaldehyde was developed by Dolbear et al. (Dolbear and Vanderlaan, 1979; Dolbear and Smith, 1977). The naphthylamine product from the enzyme action upon the substrate was coupled to NSA, forming a yellow fluorescent product. The resulted fluorescence can then be detected by flow cytometry. Using this method, Russo et al. distinguished blood leucocytes from normal and mammary carcinoma-bearing rats (Russo and Greengard, 1989).

In the kidney, the tubules of the inner cortex were most reactive for GGT by histochemical method. The outer cortex was moderately reactive. The reaction was granular, cytoplasmic and most intense at the brush border of the proximal convoluted tubule. The vascular elements in the medullar also stained. The glomeruli did not stain (Rutenberg et al., 1969).

1.4. THESIS OBJECTIVES

This study was designed to test the hypothesis that renal tubular cell exfoliation plays an important role in ARF by systematically investigating renal tubular cell shedding phenomenon and its relationship with renal function in early phase of rabbit models of ischemic and toxic ARF. The specific aims of this study were:

1) To characterize the time course of renal tubular cell shedding in the early phase of rabbit models of ARF induced by renal artery clamping, glycerol, and mercuric chloride.

2) To determine the viability of voided renal tubular cells in the different animal models.

3) To apply flow cytometry to the study of urinary cells, especially by using the proximal tubular cell marker enzymes AP and GGT to identify and quantitate this cell type in the heterogeneous urinary cell sample in ARF.

4) To study correlations between cell shedding and renal function.

CHAPTER TWO

MATERIALS AND METHODS

2.1. TIME COURSE AND VIABILITY STUDIES OF KIDNEY TUBULAR CELL SHEDDING IN 60 MIN CLAMPING, GLYCEROL, AND MERCURIC CHLORIDE INDUCED EXPERIMENTAL ARF

2.1.A. ANIMAL PREPARATION AND INDUCTION OF ARF

New Zealand white male rabbits (Vandemeer Rabbitry, Sherwood PK., Canada) weighing 3-5 kg were used for the study. Rabbits were allowed free access to tap water and purina Chow. Four groups of rabbits were designed; 1) control group; 2) renal artery clamped group; 3) glycerol-treated group; 4) mercuric chloride-treated group.

2.1.A.a. CONTROL GROUP

Rabbits were anaesthetized with intravenous sodium pentabarbital (50 mg/kg) through ear vein. The rabbit was shaved over the abdomen and medial side of the right leg. A right femoral vein cannulation was performed. A small abdominal midline incision was made for direct observation of the bladder. Urethral catheter (sylastic tube 0.030 in ID X 0.065 in OD, Dow Corning Corporation, Midland, Michigan, USA) was gently placed, paying close attention not to damage the pathway of the catheter to avoid any bleeding. The bladder was drained until empty and then washed with saline. Baseline urine and blood samples were taken through the urethral catheter and femoral vein catheter respectively. Infusion of saline was started at 9.4 cc/h and timed urine and blood collections began for every hour till hour 6.

Urine samples were collected into sterile tubes, and bladder emptying was confirmed by direct visualization. If residual urine was detected in the bladder, the urethral catheter was gently manipulated to allow the urine to flow out.

Rabbits were killed with an overdose of sodium pentobarbital at 6 h after the collection of the final urine and blood samples and the kidneys harvested.

2.1.A.b. RENAL ARTERY CLAMPING GROUP

The clamping method was based upon that of Solez et al. (1977). Rabbits were prepared as above. After anaesthesia, femoral vein cannulation,and urethral catheterization the abdominal midline incision was made. Renal arteries were separated gently from perirenal fat and renal vein. Small rubber-covered clamps were placed on left and right renal arteries. The clamps were removed from the arteries after one hour. Timing of the experiment began immediately after the clamps were placed. Infusion of saline was started after the clamps were removed and the experiment proceeded as above.

2.1.A.c. GLYCEROL TREATED GROUP

Rabbits were deprived of water overnight, anaesthetized, catheterized, and cannulated as above. The abdominal midline incision was made for bladder inspection. 50% glycerol (15 mg/kg) was then injected subcutaneously. Infusion of saline was started after the injection, and timed sample collections began.

2.1.A.d. MERCURIC CHLORIDE TREATED GROUP

Rabbits were prepared as above. After anaesthetization, catheterization, cannulation, abdominal midline incision, and baseline sample collection, 0.002% (w/v) mercuric chloride (1.5 mg/kg) was administered via ear vein. Infusion of saline was then started and timed sample collection began.

2.1.B. UREA NITROGEN AND CREATININE DETERMINATIONS

Serum and urine urea nitrogen and creatinine concentrations were determined by standard colorimetric methods (IL Mulstat® III microcentrifugal analyzer test procedure, Beckman Instruments Inc., Microbics Operations, Carlsbad, CA 92008, 1981).

2.1.C. HISTOLOGICAL METHODS

At the time of the sacrifice of the rabbit, the right kidney was harvested and immersion-fixed in 4% (v/v) formalin, and the left kidney perfusion-fixed with glutaraldehyde (0.5% and 1.5% sequentially, w/v) and stored in 3% glutaraldehyde. The kidneys were then embedded and paraffin sections were cut and stained with H and E method. The sections were then examined under a light microscope and ranked for certain histologic changes.

2.1.D. CELL COUNT AND VIABILITY ASSAY BY LIGHT MICROSCOPY AND CYTOCHEMICAL STAINING OF URINARY CELLS

Cell counts and viability study were performed using a haemocytometer and trypan blue exclusion technique.

Cytospin specimens were prepared using a Cytospin machine (Shandon Southern Products Ltd. Astmoor, Runcorn, Cheshire, England). Cytospin slides were stained by Papanicilou method.

2.1.E. CELL CULTURE

Following cell counts and viability study, urine specimens were centrifuged at 72 g for 5 min. The supernatant was discarded and the pellet resuspended in sterile culture medium containing Hams F12:DMEM (Gibco, Grand Island, N.Y., USA) with 10mM Hepes, 10% (w/v) fetal serum, penicillin (50 unit/ml) (Gibco), streptomycin (50 ug/ml) (Gibco), and fungizone (1.25 ug/ml) (Gibco). Cell suspension was then plated in small plastic culture dishes coated with collagen at a concentration of 5×10^4 to 5×10^5 viable tubular cell/ml. Cultures were maintained at 37°C in 5% CO_2 atmosphere and refed

every 2 to 3 days with the culture medium of tapering amounts of fetal calf serum (10%, 5%, 2.5%, 0%, v/v, Gibco). At fetal calf serum concentrations of 5% or lower, the medium was supplemented with insulin (5 ug/ml,), transferin (5 ug/ml,),sodium selenite (5 X 10^{-8} M, Sigma Chemical Company, ST. Louis, MO, USA), hydrocortisone(5 X 10^{-8} M, Sigma), fungizone (1.25 ug/ml), penicillin (50 unit/ml), and streptomycin (50 ug/ml). Cultures were monitored by phase contrast microscope at each feeding, and presence or absence of cell attachment or growth, and extent of confluence of established monolayers noted.

Monolayers reaching confluence were trypsinized and subculture attempted. Culture medium was removed from the culture dish, and the dish flooded with trypsin EDTA (Gibco) in balanced salt solution without calcium and magnesium. Culture dishes were then placed in the incubator for 15 min, resulting in near total cell detachment, with 90+% viability. Cells were then diluted to 1-5 X 10^5 cell/ml in culture medium with 10% fetal calf serum, seeded into coated culture dishes, and observed as in primary cultures.

2.1.F. DATA ANALYSIS

Data were analyzed using the CLINFO computer system (NIH RR 00035). The Kruskal-Wallis one-way analysis of variance for nonparametric data and Spearman rank correlation were used.

2.2. CHARACTERIZATION OF VOIDED URINARY CELLS BY FLOW CYTOMETRY

2.2.A. ANIMAL PREPARATION AND INDUCTION OF ARF

Four groups of rabbis were designed for this study; 1) control group; 2) 60 min renal artery clamped group; 3) 45 min renal artery clamped group; and 4) 30 min renal artery clamped group. Clamping and experimental procedures were performed as described previously except that saline was infused at greater speed (about 30 cc/h) in order to increase urine output and cell number for FC analyses. Urine and blood samples were collected every 2 h instead of every 1 h.

2.2.B. VOIDED URINARY CELL PREPARATION FOR FC ANALYSIS

Urine samples were centrifuged at 72 g for 5 min. The supernatant was discarded and the pellet resuspended in Hams F12:DMEM culture medium as previously described. Samples were then kept in a 37°C incubator with 5% CO_2 atmosphere overnight. Samples were then filtered through a nylon mesh filer (52 u in diameter, Spectrum, USA). Large aggregates in the sample were sieved out to prevent clotting of the flowcytometer. The samples were then washed with Hanks buffer, residual fluorescent materials which might be left in the sample from the culture medium were cleaned out, eliminating the interference of these materials with the analyses of the desired fluorescent signals.

2.2.C. FLUORESCENT AP STAINING

Fluorescent alkaline phosphatase staining was based upon the method of Dolbear's (1980). Modification was made by adding NaCl to the tris-borate buffer to increase osmolality from 173 mOsm/kg to 277 mOsm/kg in order to prevent cell lysis which might result from the low osmolality of the incubation solution of the original method.

Cells were incubated in the substrate solution consisting of 0.1 M tris-borate buffer, pH 9.0, 5 mM $MgCl_2$, 1.0 mM naphthol AS-BI phosphate (Sigma), and 0.2 mM fast red TR salt (Sigma), at 37°C for 5 min. Cells were then washed with 0.2 M sodium acetate buffer, pH 5.0, and resuspended in Hanks buffer.

2.2.D. CYTOCHEMICAL AP STAINING

Cytochemical AP staining of cytospin slides was done using the same method as for fluorescent AP staining. Cells positive for AP stained red.

2.2.E. FLUORESCENT GGT STAINING

Fluorescent cytochemical staining of GGT was based on the method of Russo and Greengard (1989). The standard assay mixture contained 1 mM gamma-glutamyl-4-methoxy-2-naphthylamide (gamma-glu-MNA) (Polysciences INC., Warrington, PA.), 1 mM 5-nitrosalicylaldehyde (NSA) (Eastman Kodak Company, Rochester, N.Y., USA), 0.1 M tris-aminomethane, pH 8.0, 80 mM glycylglycine, and 60 mM NaCl.

The substrate, gamma-glu-MNA, was dissolved in a small volume of 1:1 dimethylformamide (DMF) (Fisher Scientific, USA) and 1N HCl. The coupling agent, NSA, was dissolved in DMF. The final concentration of DMF in the assay mixture was kept below 3%.

2.2.F. FLUORESCEIN DIACETATE STAINING

FDA staining was based on the method of Rotman and Papermaster (1966). 10 uM/ml FDA (Sigma) was added to the cell suspension 15 min before FC analysis.

2.2.G. PREPARATION OF RABBIT BLOOD CELLS

Rabbit white blood cells and red blood cells were used as references for the characterization of the voided urinary cells. Rabbit blood was obtained through the femoral vein cannula after the final blood sample was taken.

2.2.G.a. SEPARATION OF RABBIT LEUCOCYTE

The separation of rabbit leucocytes was based on the method of Russo and Greenguard's (Russo and Greengard 1989). The whole blood was mixed with two volumes of 1.5% (w/v) Dextran T-500 (Pharmacia LKB, Biotechnology AB, Uppsala, Sweden) in 0.9% (w/v) NaCl solution and allowed to stand for 30 min at room temperature in a centrifuge tube. RBCs settled at the bottom of the tube. The upper leucocyte layer was

carefully collected, diluted with the same volume of 0.9% (w/v) NaCl solution and centrifuged. Remaining RBCs were lysed with 0.2% (w/v) NaCl. After restoring osmolality with 1.8% (w/v) NaCl, the suspension was centrifuged and the sedimented WBCs were treated with urine supernatant (saved from urine sample) for 2 h at 37°C in order to provide the same conditions for the leucocytes as those which the voided cells in the urine encountered before sample processing. WBCs were then centrifuged and resuspended in Hams F12:DMEM culture medium as previously described. WBCs were also stored in a 37°C incubator with 5% CO_2 atmosphere overnight.

AP, GGT, and FDA staining of WBCs were preformed using the same methods as those for urine cell staining.

2.2.G.b. RABBIT RED BLOOD CELL PREPARATION

Rabbit whole blood was used for the analyses of RBCs because the dominant cell type of whole blood was RBC. The whole blood was diluted to 1 X 10^4 cell/ml with saline before FC analysis.

2.2.H. FLOW CYTOMETRIC ANALYSES

An EPICS V cytometer (Coulter Electronics, Hialeah, Florida) equipped with an argon laser (Innova 90 Series, Coherent Laser Products Division,Polo Alto, CA) was used for this study.

2.2.H.a. FALS DATA COLLECTION

For FALS data collection, unstained samples were excited at 488 nM and the scattered light filtered through a 570 and a 505 long pass filter. FALS distribution of RBCs was used as the reference for setting the gate to eliminate debris and RBCs for data collection of WBC and urine cell samples.

2.2.H.b. FDA DATA COLLECTION

Excitation of the flowcytometer was set at 488 nM for FC analysis of FDA stained samples. The emission was filtered through a 570 and a 505 long pass filter. FALS was measured concurrently with the fluorescence. RBCs were gated out for data collections of WBC and urine cell samples as above.

2.2.H.c. FLUORESCENT AP ACTIVITY DATA COLLECTION

Fluorescent product resulted from AP staining was quantified in single cells by the flowcytometer with the excitation set at 514 nM. The emitted light was filtered through a 610 long pass and a 635 band pass filter. FALS data were collected simultaneously, and gating was performed as above.

2.2.H.d. FLUORESCENT GGT ACTIVITY DATA COLLECTION

GGT stained samples were excited at 457 nM. A 570 long pass filter was used. FALS data were also concurrently collected and debris and RBCs were gated out using the same

method as above.

Fluorescent microscopy for FDA, AP, and GGT stained samples was performed using a Leitz Dialux 22 fluorescent microscope (Ernst Leitz Wetzalar GMBH, Germany).

2.2.I. DATA ANALYSIS

Data were analyzed using STATS-2 on an IBM computer. Nonparametric statistics and Spearman rank order correlation were used.

CHAPTER THREE

RESULTS

3.1. TIME COURSE AND VIABILITY STUDIES OF EXFOLIATED KIDNEY CELLS IN 60 MIN RENAL ARTERY CLAMPING, GLYCEROL AND MERCURIC CHLORIDE INDUCED EXPERIMENTAL ARF

3.1.A. RENAL FUNCTION

Urea nitrogen and creatinine clearance were significantly depressed beginning in the first h for the clamped rabbits and from the third h on for the other two experimental groups.

Mean serum urea nitrogen and creatinine concentrations over the 6-h experimental period were significantly elevated (Table 2).

Mean urea nitrogen and creatinine clearances over the 6-h experimental period in all experimental groups were significantly depressed compared to control group (Table 2).

Renal failure tended to be oliguric in the clamped group, especially initially, and polyuric in glycerol-treated and mercuric chloride-treated rabbits (Table 2).

3.1.B. CELL NUMBERS AND VIABILITIES

On wet LM preparation of urine samples stained with trypan blue, viable cells appeared clear (Plate 1). Renal tubular cells were recognized as cells with round nuclei and slightly larger in size than granulocytes which were also present. Granulocytes characteristically showed two or more coupled nuclei. Nonviable cells were stained blue and often were recognized as renal tubular cells. Nonviable granulocytes were rarely seen.

The quantification of dead and live renal tubular cells in urine analyzed by light microscopy and trypan blue exclusion method are shown in Table 3.

The total cell number and total viable cell number voided over the 6-h experiment in all experimental groups except total voided cell number in clamped group were significantly greater compared to control group.

The concentrations of total voided cells and total voided live cells were significantly higher in all experimental groups compared to control group.

Mean percent viability was low in control and glycerol-treated animals and significantly higher in clamped and mercuric chloride-treated animals compared to control group.

Papanicoloa (PAP) method stained cytospin preparation of voided urine confirmed significant numbers of voided renal tubular cells in most experimental animals (Plate 2.).

TABLE 2

MEAN SERUM UREA NITROGEN AND CREATININE CONCENTRATIONS, MEAN UREA NITROGEN AND CREATININE CLEARANCES, AND TOTAL URINE OUTPUT IN 60 MIN RENAL ARTERY CLAMPING, GLYCEROL-, AND MERCURIC CHLORIDE-TREATED ANIMALS

		Mean[a]				Accumulated[a]
Animal Model	N	SUN (mg/dl)	SCR (mg/dl)	C_{UN} (ml/min)	C_{CR} (ml/min)	Urine Output (ml)
Control	10	18±1	1.0±0.1	3.0±0.3	8.0±1.7	13±1
clamp	10	27±2*	2.5±0.1*	0.1±0*	0.2±0.1*	6±3*
glycerol	10	26±1*	1.6±0.2*	1.2±0.2*	3.1±0.4*	49±7*
$HgCl_2$	11	21±2	1.5±0.2*	1.3±0.2*	2.5±0.4*	32±7*

* Significantly different from control group.

a=data illustrate mean ± SE
SUN=serum urea nitrogen
SCR=serum creatinine
C_{UN}=urea nitrogen clearance
C_{CR}=creatinine clearance

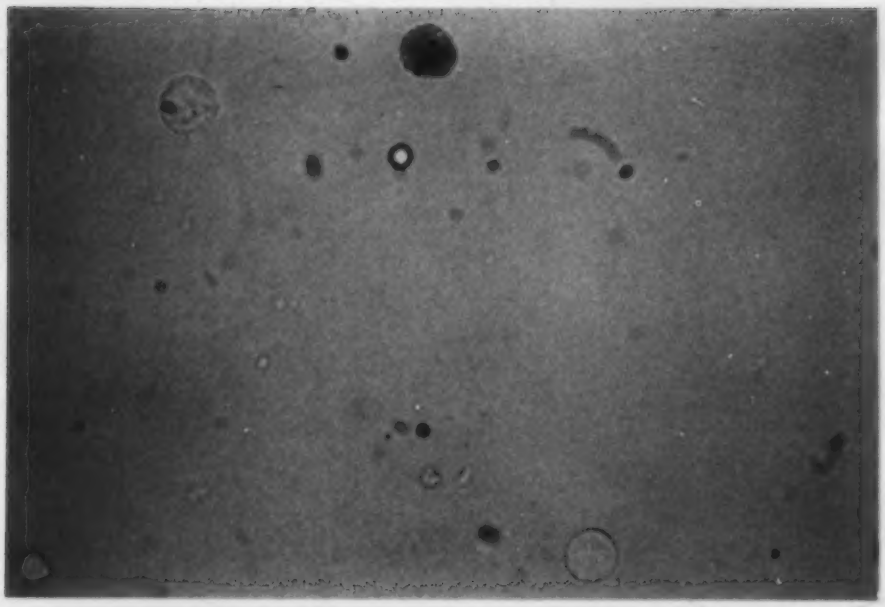

PLATE 1. Trypan blue stained urinary renal tubular cells (100X).

TABLE 3

TOTAL CELL NUMBERS, CONCENTRATIONS, AND VIABILITIES OF VOIDED TUBULAR CELLS IN 60 MIN RENAL ARTERY CLAMPING, GLYCEROL-, AND MERCURIC CHLORIDE-TREATED ANIMALS

		Accumulated[a]			Concentrations ($X10^{-4}$)[a] (cell/ml)	
Animal Model	N	Total Cells ($X10^{-6}$)	Total Viable ($X10^{-6}$)	% Viable	Total Cells	Viable Cells
Control	10	0.2±0	0±0	7±5	1.4±0.2	0.1±0.1
Clamp	10	4.7±1.3	1.8±0.6*	38±6*	122.5±38.9*	42.6±15.3*
Glycerol	10	39.3±13.7*	2.8±1.1*	11±3	74.8±17.3*	5.7±1.6*
$HgCl_2$	11	25.5±10.7*	15.5±9.0*	51±8*	83.2±22.1*	37.6±11.3*

* $P<0.05$ compared to control animal

a=data illustrate mean ± SE

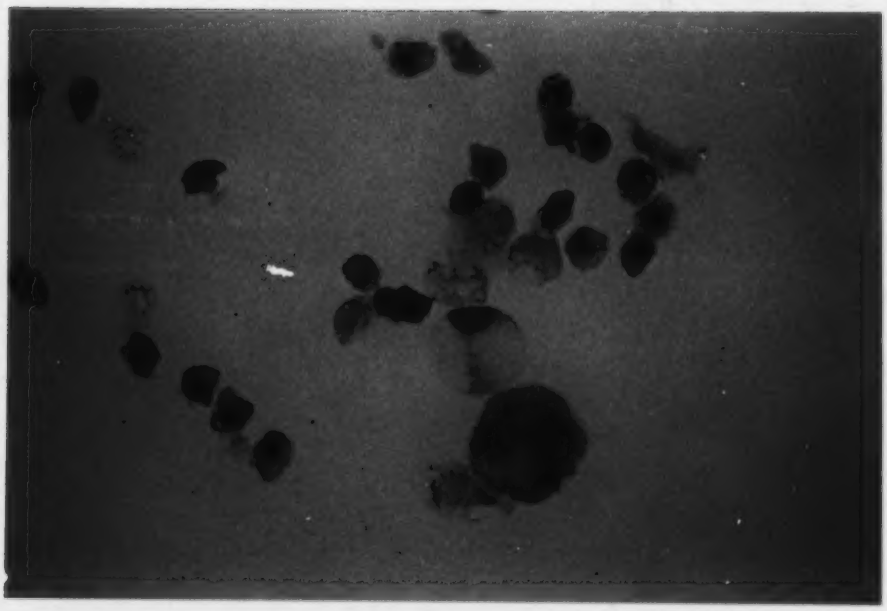

PLATE 2. Urinary renal tubular cells on cytospin preparation stained by PAP method (45X).

3.1.C. PATHOLOGICAL CHANGES

3.1.C.a. RENAL ARTERY CLAMPING MODEL

Extensive necrosis in the outer medulla with pyknosis karyorhexis and loss of nuclear staining was found. In renal cortex changes were largely limited to nuclear pyknosis in proximal tubular cells in the medullary rays and detachment of these cells with formation of collections of free luminal tubular cells. Tubular casts formation was not particularly prominent in the cortex at 6 h, but some tubular cells with pyknotic nuclei were incorporated into tubular casts.

3.1.C.b. GLYCEROL MODEL

Morphologic changes were less severe than those observed in the clamping model. Scattered necrosis of proximal and distal tubules were seen, but the main change observed was the presence of hyaline and pigmented tubular casts.

At this early time point, some of the animals' renal dysfunction was likely to be prerenal due to a combination of the overnight dehydration and the intravascular volume depletion which occurs as a direct consequence of glycerol injection. This acute prerenal component of the renal dysfunction would not be expected to have a morphological counterpart.

3.1.C.c. MERCURIC CHLORIDE MODEL

Morphologic changes consisted mainly of nuclear pyknosis and cell detachment in the cells of the S3 segment of the proximal tubule. In contrast to the clamping model in which marked outer medullary changes were seen, few medullary alterations were seen in mercuric chloride group.

Results of ranking revealed highly significant differences between experimental and control animals (Table 4).

3.1.D. CELL CULTURE

Culture of shed cells was attempted in 3 clamped rabbits, all grew to confluence and were successfully passaged. Three culture attempts of specimens from mercuric chloride-treated animals yielded focal monolayers on one specimen only. There was no growth in vitro of the single specimen attempted on glycerol-treated rabbits.

3.1.E CORRELATIONS AMONG VOIDED CELL NUMBERS, PATHOLOGICAL CHANGES, AND RENAL FUNCTION

Table 5 demonstrates the correlations among cell shedding, renal function and pathological changes.

A significant correlation existed between total and total viable tubular voided cells, and total urine output in clamped animals ($r=+0.673$ and $r=+0.697$ respectively, $0.01<P<0.05$).

A significant negative correlation was present between total voided live cells or percent viable cells, and mean

TABLE 4

MEAN RANKS OF CELL NECROSIS ON RENAL HISTOLOGICAL EXAMINATION IN 60 MIN RENAL ARTERY CLAMPING, GLYCEROL-, AND MERCURIC CHLORIDE-TREATED ANIMALS

Animal Model	Mean Rank[a]
Control	5.0±0
Clamping	35.5±0.9*
Glycerol	14.2±1.0*
$HgCl_2$	24.3±1.2*

* $P<0.05$ compared to control group

a = data illustrate mean ± SE

TABLE 5

CORRELATIONS AMONG CELL SHEDDING, RENAL FUNCTION, AND PATHOLOGICAL CHANGES IN 60 MIN RENAL ARTERY CLAMPING, GLYCEROL-, AND MERCURIC CHLORIDE-TREATED ANIMALS (Spearman's rank correlation)

1.	Total voided tubular cells and total urine output in clamped animals	r=+0.673	0.01<P<0.05
2.	Total viable voided tubular cells and total urine output in clamped animals	r=+0.697	0.01<P<0.05
3.	Total voided live cells and mean serum creatinine in $HgCl_2$-treated animals	r=-0.6485	0.01<P<0.05
4.	Percent viable cells and mean serum creatinine in $HgCl_2$-treated animals	r=-0.7576	0.01<P<0.05
5.	Total live cells and mean creatinine clearance in $HgCl_2$-treated animals	r=+0.8545	P<0.01
6.	Mean percent viable cells and mean creatinine clearance in $HgCl_2$-treated animals	r=+0.8182	P<0.01
7.	Tubular necrosis ranking and mean SUN in $HgCl_2$-treated animals	r=+0.7882	P<0.01
8.	Tubular necrosis ranking and mean urea nitrogen clearance in $HgCl_2$-treated animals	r=-0.6270	P<0.01
9.	Tubular necrosis ranking and mean urea nitrogen clearance in glycerol-treated animals	r=-0.7029	P<0.01
10.	Tubular necrosis ranking and mean concentration of viable voided cells in glycerol-treated animals	r=+0.7113	0.01<P<0.05

serum creatinine in the $HgCl_2$-treated animals ($r=-0.6485$ and $r=-0.7576$ respectively, $0.01<P<0.05$).

There was a significant positive correlation between total live or mean percent viable cells, and mean creatinine clearance ($P<0.01$, $r=+0.8545$ and $r=+0.8182$ respectively) in $HgCl_2$-treated animals.

No significant correlations were found between voided cells and functional parameters in the glycerol-treated rabbits.

Tubular necrosis ranking correlated with mean serum urea nitrogen concentration ($P<0.01$, $r=+0.7882$)) and mean urea nitrogen clearance ($.01<P<.05$, $r=-0.6270$) in $HgCl_2$-treated rabbits.

Tubular necrosis ranking negatively correlated with mean urea nitrogen clearance ($0.01<P<0.05$, $r=-0.7029$) and mean concentration of viable voided cells ($0.01<P<0.05$, $r=+0.7113$) in the glycerol-treated rabbits.

3.2. FLOW CYTOMETRIC ANALYSES OF VOIDED URINARY CELLS IN 60 MIN, 45 MIN, AND 30 MIN CLAMPING MODELS OF ARF

3.2.A. RENAL FUNCTION

Urine output tended to be polyuric in 60 min and 45 min clamping models beginning at hour 2 (Table ., Fig. 6). Increased saline infusion rate probably played a role for the difference of the urine output between the previous 60 min clamped animals (Table 2, p.42) and the present ones.

Urea nitrogen and creatinine clearances for all experimental groups were significantly depressed, beginning at hour 2 (Table 6, Fig. 7, 8).

Mean urea nitrogen concentrations for 60 min and 30 min clamping groups were significantly elevated (Table 6). Serum creatinine concentration was significantly increased for 60 min clamping group (Table 6).

3.2.B. CELL NUMBERS AND VIABILITIES STUDIED BY LIGHT MICROSCOPY

Accumulated total and total viable cell numbers were significantly greater in all experimental groups than in control group (Table 7). Cell shedding started at hour 2 (Fig. 9, 10).

Viabilities of voided renal tubular cells determined by trypan blue exclusion test ranged from 41% to 61% for the experimental groups (Table 7).

Concentrations of total voided cells and total live cells in 60 min and 45 min clamped groups were significantly higher compared to control group (Table 7). There were no significant difference between the concentrations of total voided cell and total voided live cell of 45 min clamping group and control group.

TABLE 6

MEAN SERUM UREA NITROGEN AND CREATININE CONCENTRATIONS , UREA NITROGEN AND CREATININE CLEARANCES, AND TOTAL URINE OUTPUT IN 60 MIN, 45 MIN, AND 30 MIN RENAL ARTERY CLAMPING ANIMALS

Animal Model	N	Mean[a] SUN (mg/dl)	SCR (mg/dl)	C_{UN} (ml/min)	C_{CR} (ml/min)	Total Urine Output[a] (ml)
Control	7	16.5±1.3	1.5±0.3	3.3±0.4	6.2±1.2	13.1±1.8
60 min	9	26.9±1.9*	3.0±0.4*	0.3±0.1*	0.5±0.2*	29.8±13
45 min	6	19.3±1.6	2.1±0.3	0.8±0.2*	1.7±0.4*	44.3±12
30 min	8	22.9±1.0*	2.1±0.2	0.8±0.3*	2.4±0.6*	14.9±5.0

*P<0.05 compared to control animals

a=data illustrate mean ± SE
SUN=serum urea nitrogen
SCR=serum creatinine
C_{UN}=urea nitrogen clearance
C_{CR}=creatinine clearance
60 min = 60 min renal artery clamping model
45 min = 45 min renal artery clamping model
30 min = 30 min renal artery clamping model

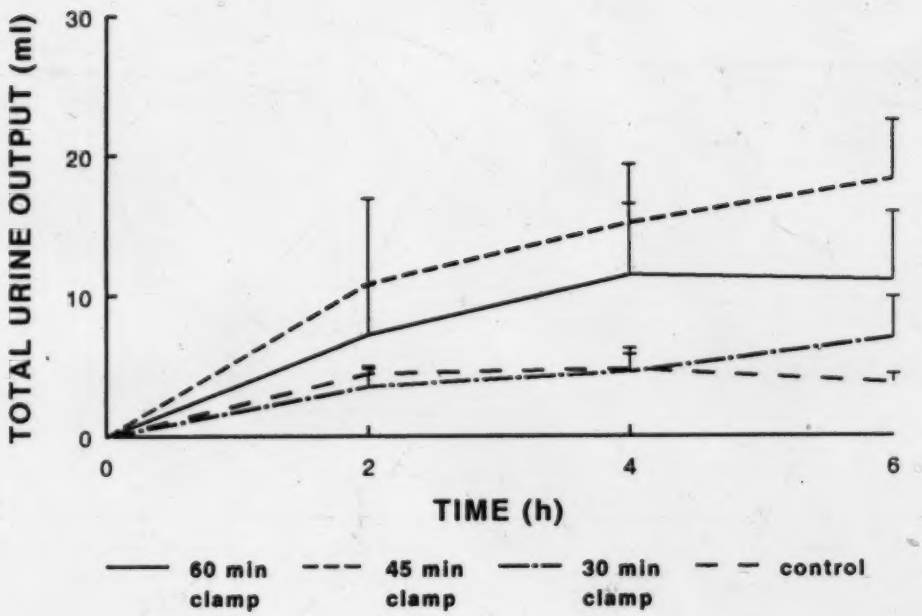

FIGURE 6. Time course of total urine output in 60 min, 45 min, and 30 min renal artery clamping animals.

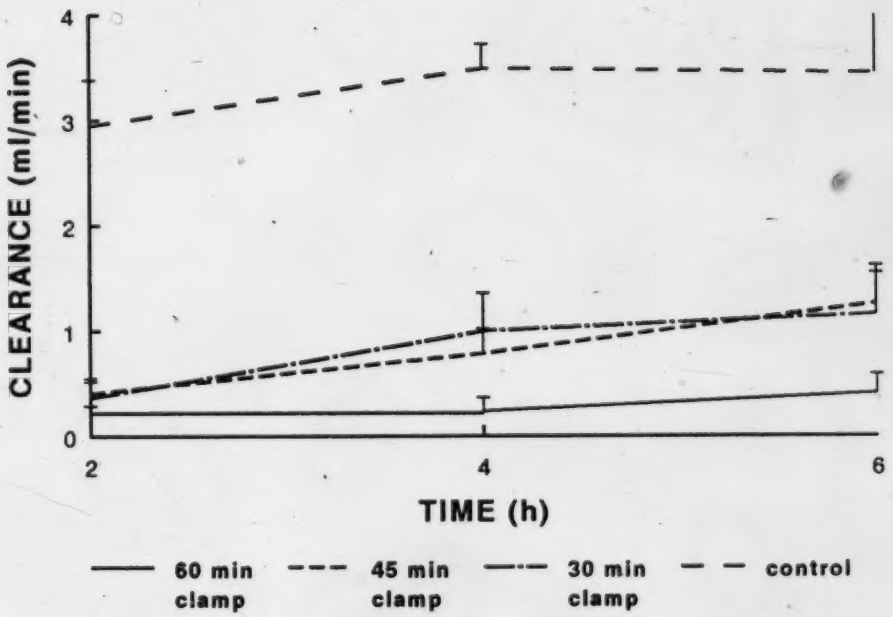

FIGURE 7. Time course of urea nitrogen clearance in 60 min, 45 min, and 30 min renal artery clamping animals.

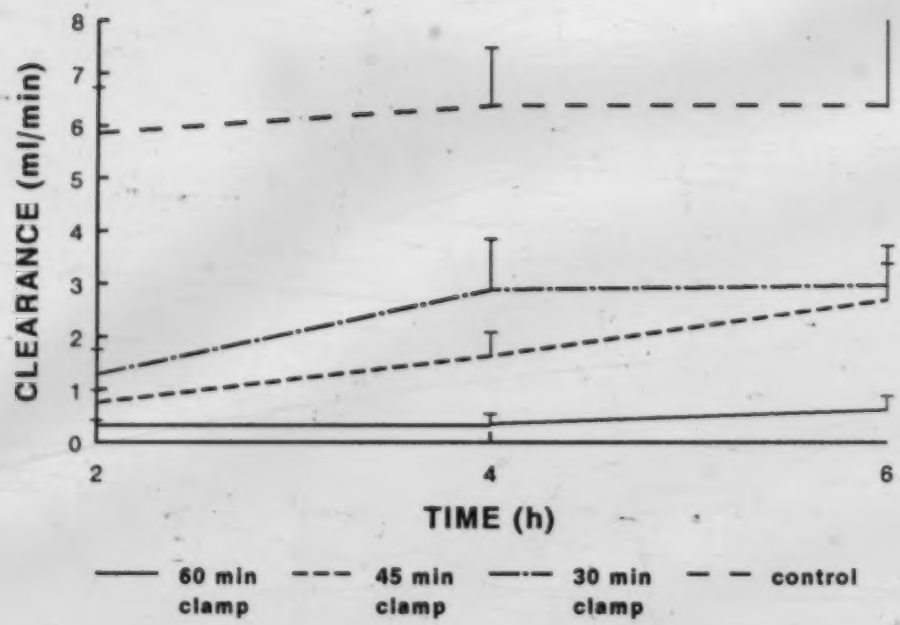

FIGURE 8. Time course of creatinine clearance in 60 min, 45 min, and 30 min renal artery clamping animals.

TABLE 7

TOTAL CELL NUMBERS, CONCENTRATIONS, AND VIABILITIES OF VOIDED TUBULAR CELLS IN 60 MIN, 45 MIN, AND 30 MIN RENAL ARTERY CLAMPING ANIMALS

Animal Model	N	Accumulated ($X10^{-6}$)[a] Total Viable	Accumulated ($X10^{-6}$)[a] Total Cells	% Viable	Concentration ($X10^{-6}$)[a] (cell/ml) Total Viable	Concentration ($X10^{-6}$)[a] (cell/ml) Total Cells
Control	7	0.9±0.5	0.8±0.5	46±14	0.1±0.0	0.1±0.0
60 min	9	12.5±3.9*	8.5±3.3*	56±5	2.9±1.6	2.0±1.2
45 min	6	10.2±1.6*	5.9±1.1*	61±9	0.2±0.1	0.3±0.1
30 min	8	9.5±2.0*	3.5±0.9*	41±7	0.9±0.7	1.7±0.8

*P<0.05

a=data illustrate mean ± SE
60 min = 60 min renal artery clamping model
45 min = 45 min renal artery clamping model
30 min = 30 min renal artery clamping model

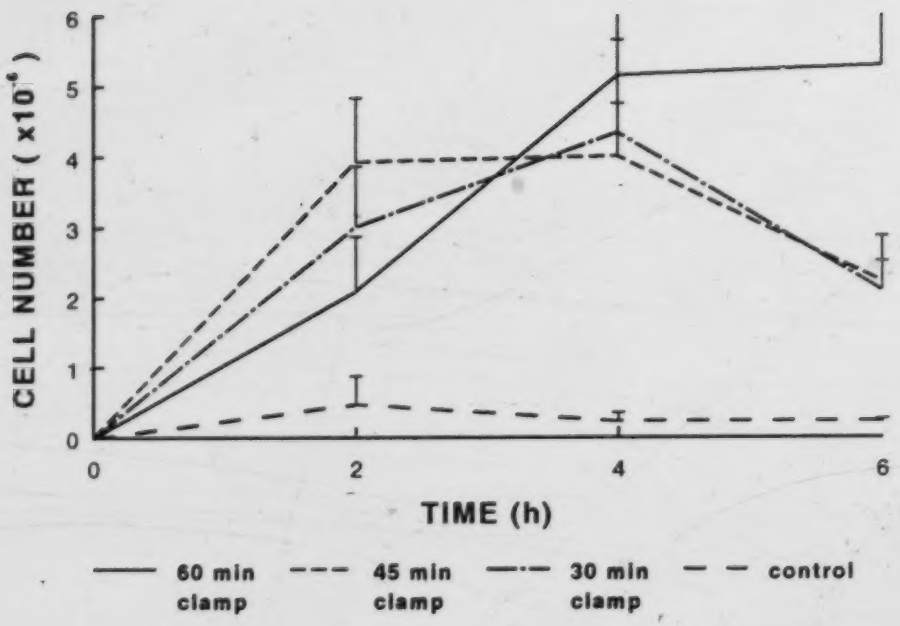

FIGURE 9. Time course of total renal tubular cell shedding studied by LM in 60 min, 45 min, and 30 min renal artery clamping animals.

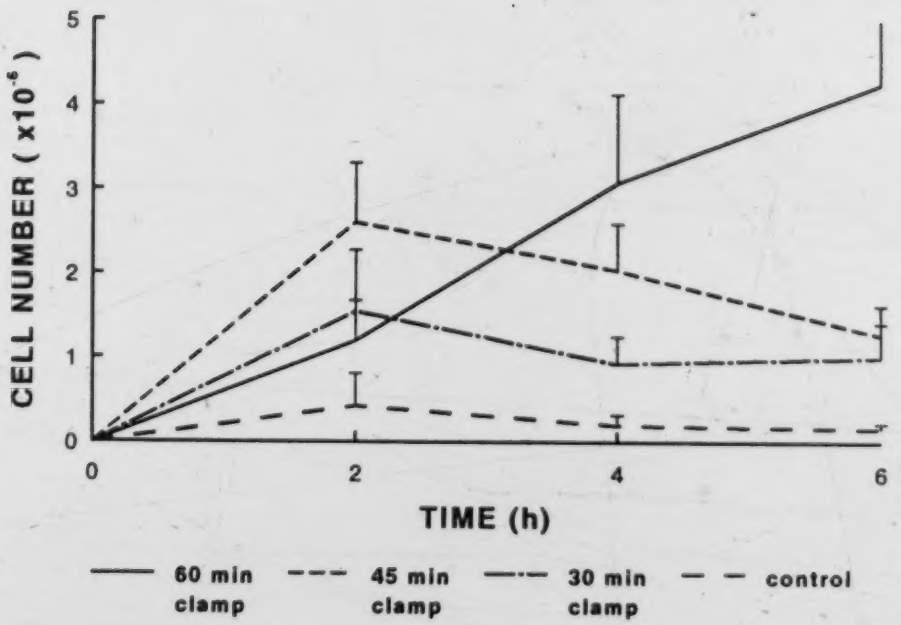

FIGURE 10. Time course of total live renal tubular cell shedding studied by LM in 60 min, 45 min, and 30 min renal artery clamping animals.

3.2.C. FLOW CYTOMETRIC CHARACTERIZATION OF VOIDED URINARY CELLS AND RABBIT BLOOD CELLS

3.2.C.a. ASSESSMENT USING FALS

3.2.C.a.1) RABBIT RED BLOOD CELLS

When rabbit whole blood was analyzed by flow cytometry, the intensity distribution of light scattered at small forward angle (FALS) generated a histogram with the major peak located near the Y axis (low FALS intensity) (Fig. 11, A). Since the dominant cell type of a whole blood sample is RBCs this major peak most likely represented the rabbit RBC population.

3.2.C.a.2) RABBIT WHITE BLOOD CELLS

Separated rabbit leucocytes prepared by Dextran method produced an unipeak FALS histogram (Fig. 11, C). The peak was located farther away from the Y axis than that produced by RBCs. This result was in agreement with the fact that leucocytes are larger cells than red blood cells, hence they have higher FALS intensity. No subpopulations of WBCs could be detected by FALS alone in this study.

3.2.C.a.3) VOIDED URINARY CELLS

A reproducible histogram with no clear definition of subpopulations of cells was obtained when urinary cells were analyzed for FALS distribution after gating off the RBCs and cell debris (Fig. 11, B). The majority of cells had the same FALS intensity as or slightly higher FALS intensity than that of WBCs.

3.2.C.b. ASSESSMENT USING FDA

3.2.C.b.1) RABBIT RED BLOOD CELLS

One parameter fluorescence histogram of rabbit whole blood preparation stained with FDA showed a unipeak distribution (Fig. 12, A). The peak was most likely produced by RBCs and located close to the Y axis indicating the corresponding population of cells had low fluorescence intensity resulted from FDA staining (FDA fluorescence).

Two parameter dot plot of whole blood preparation analyzed for FDA fluorescence revealed one cell population with both low FALS and low FDA fluorescence (Fig. 13, A). This cell population most likely represented RBCs in the whole blood sample.

3.2.C.b.2) RABBIT WHITE BLOOD CELLS

When rabbit WBC preparations were analyzed for FDA fluorescence, a unipeak histogram was always obtained (Fig. 12, C). The peak was located towards the right side of the histogram, having high FDA fluorescence. No subpopulations of WBCs could be demonstrated by FDA fluorescence alone.
Two parameter dot plot of WBC FDA fluorescence showed a better resolution of different cell populations with variable

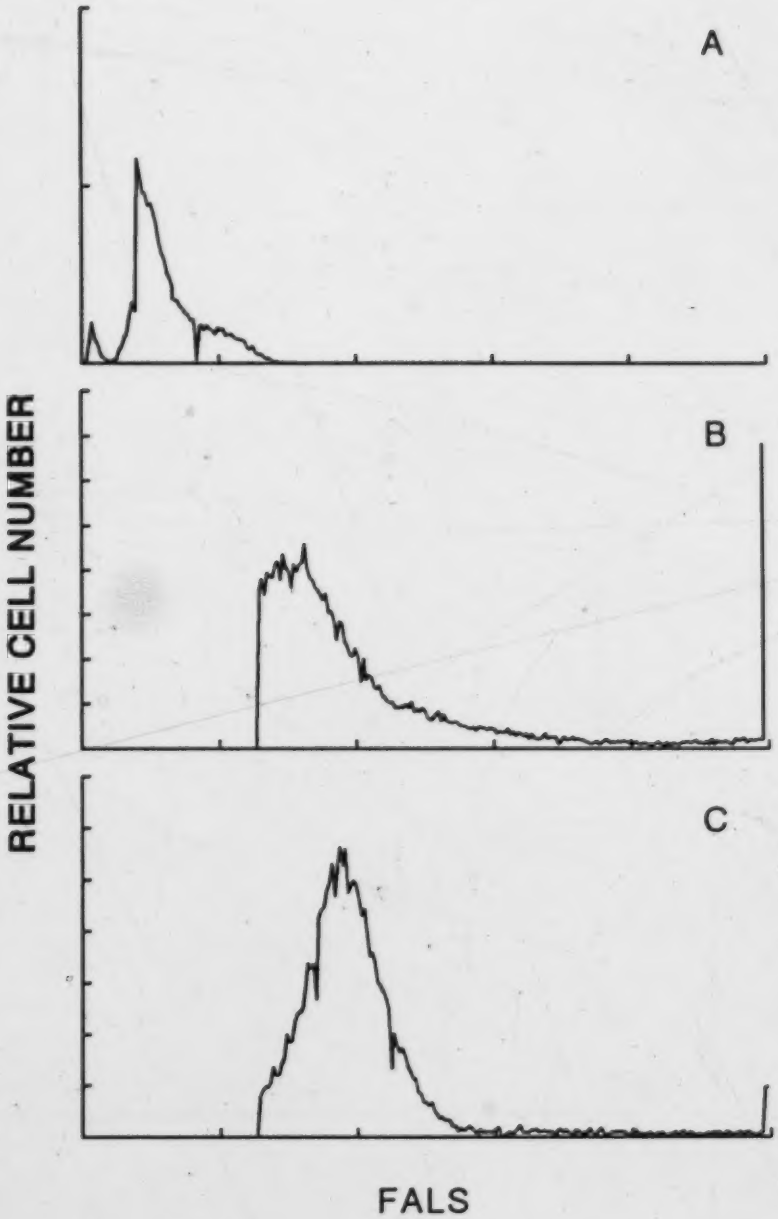

FIGURE 11. One parameter histogram of FALS of rabbit RBCs (A), urinary cells (B), and WBCs (C).

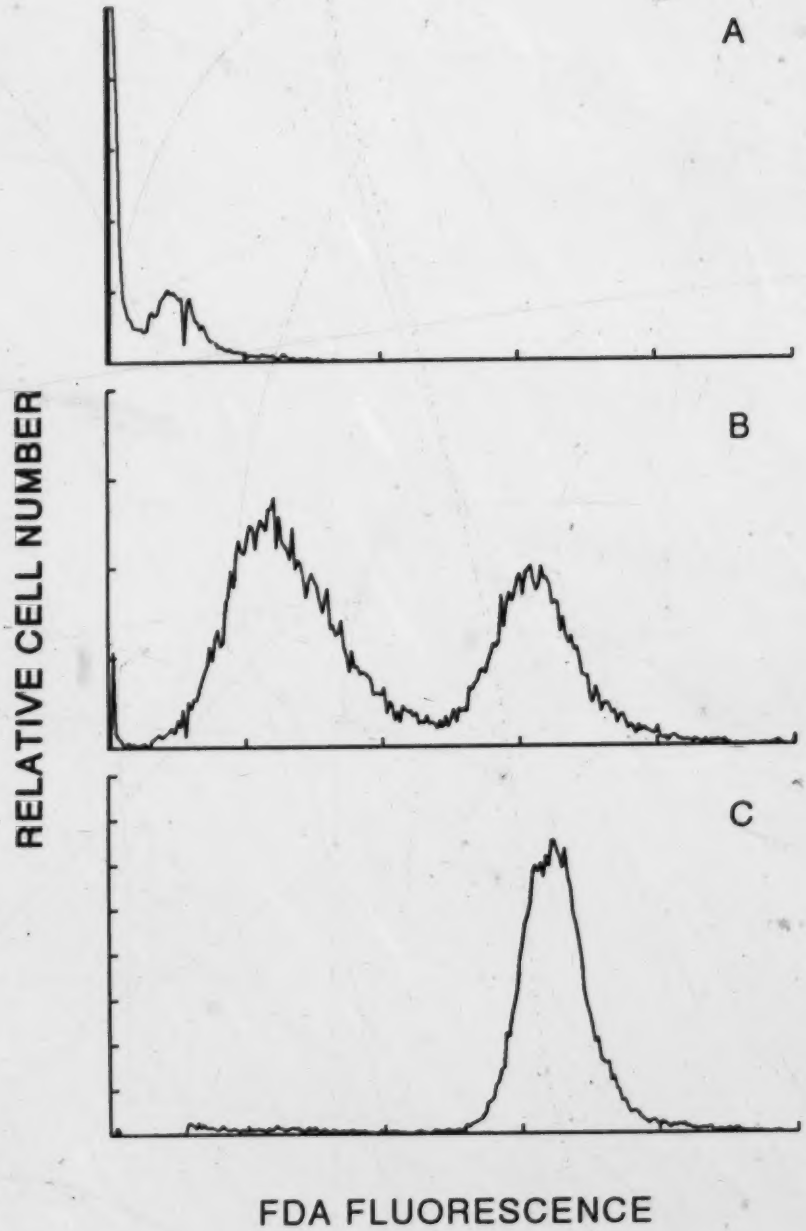

FIGURE 12. One parameter histogram of FDA fluorescence of rabbit RBCs (A), urinary cells (B), and WBCs (C).

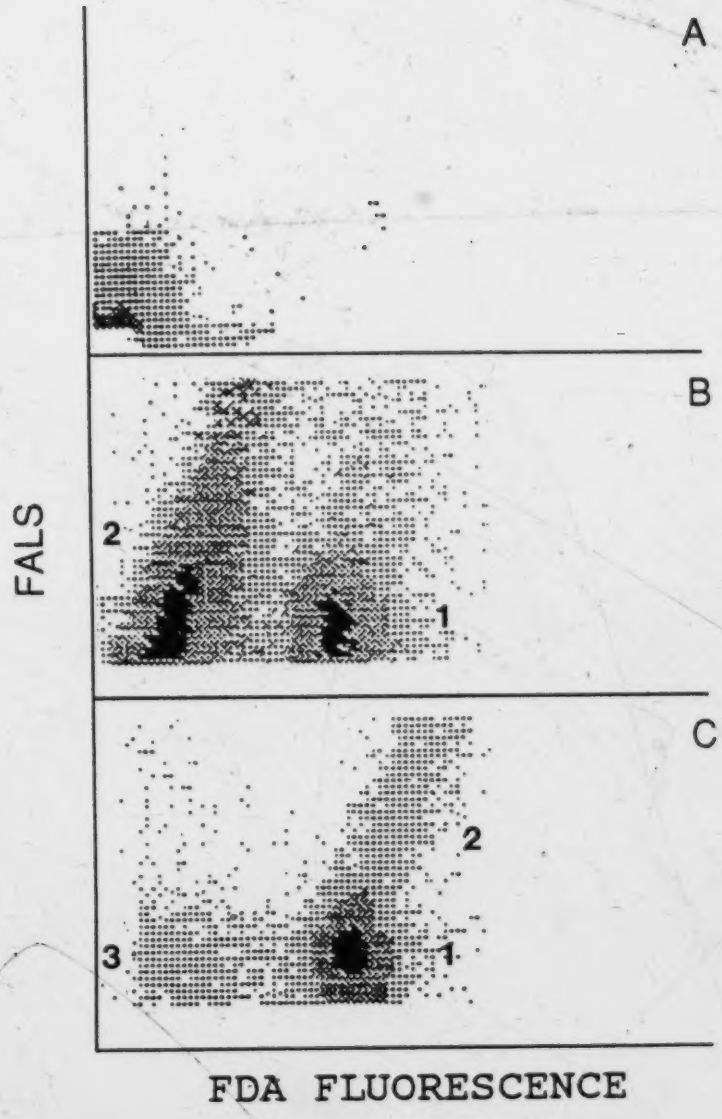

FIGURE 13. Dot plot of FDA fluorescence vs FALS of rabbit RBCs (A), urinary cells (B), and WBCs (C).

nonspecific esterase activities (Fig. 13, C). Three populations of cells could be distinguished. Population 1 had a higher FALS intensity than that of RBCs and a high FDA fluorescence intensity. Population 2 had even higher FALS and slightly higher FDA than population one. Population 3 had the same FALS intensity as and a lower FDA fluorescence than population 1. These three populations most likely represented granulocytes with high nonspecific esterase activity, monocytes, and granulocytes with low nonspecific esterase activity respectively.

3.2.C.b.3) VOIDED URINARY CELLS

Analysis of cells shed in urine for FDA fluorescence gave a histogram with two peaks (Fig. 12, B). The peak on the right side of the histogram with higher FDA fluorescence corresponded with that of WBCs. The other peak had a FDA fluorescence intensity higher than that of RBCs but lower than that of WBCs. This cell population probably represented renal tubular cells in the urine.

Two parameter dot plot of urine cells' FDA fluorescence distribution revealed two major cell populations (Fig. 13, B). Population 1 corresponded with granulocytes with high FDA fluorescence. The FDA fluorescence intensity of population 2 was lower than that of population 1 but higher than that of RBCs. This population of cells had similar and slightly higher FALS intensity than that of WBCs and probably represented renal tubular cells.

Under fluorescent microscopic examination, nonspecific esterase positive cells stained green with FDA (Plate 3).

3.2.C.c. ASSESSMENT USING ALKALINE PHOSPHATASE ACTIVITY

3.2.C.c.1) RABBIT WHITE BLOOD CELLS

When the fluorescence distribution resulted from AP staining (AP fluorescence) of WBCs was analyzed, two peaks could be detected on the one parameter fluorescence histogram (Fig. 14, A). The major peak represented a cell population with higher AP fluorescence than the minor one.

Two parameter dot plot of WBC AP fluorescence distribution showed a distinction of three cell populations (Fig. 15, A). Population 1 was the dominant population and had high AP fluorescence. Population 2 had similar AP fluorescence as but a higher FALS intensity than population 1. Population 3 had lower AP fluorescence than and the same FALS intensity as population 1. These three populations most likely represented the rabbit neutrophils, macrophages, and basophils and eosinophils respectively.

3.2.C.c.2) VOIDED URINARY CELLS

Analysis of AP fluorescence distribution of urine cells generated a reproducible histogram with three peaks (Fig. 14, B). The peak in the middle and the peak to the left

PLATE 3. Urinary cells stained with FDA (10X).

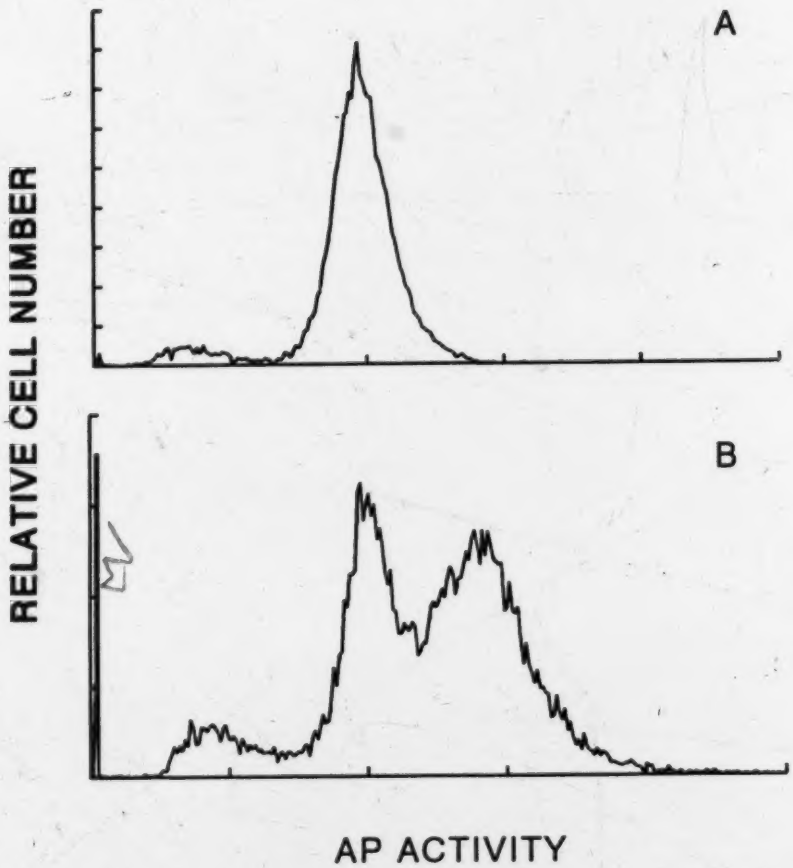

FIGURE 14. One parameter histogram of alkaline phosphatase activity of rabbit WBCs (A) and urinary cells (B).

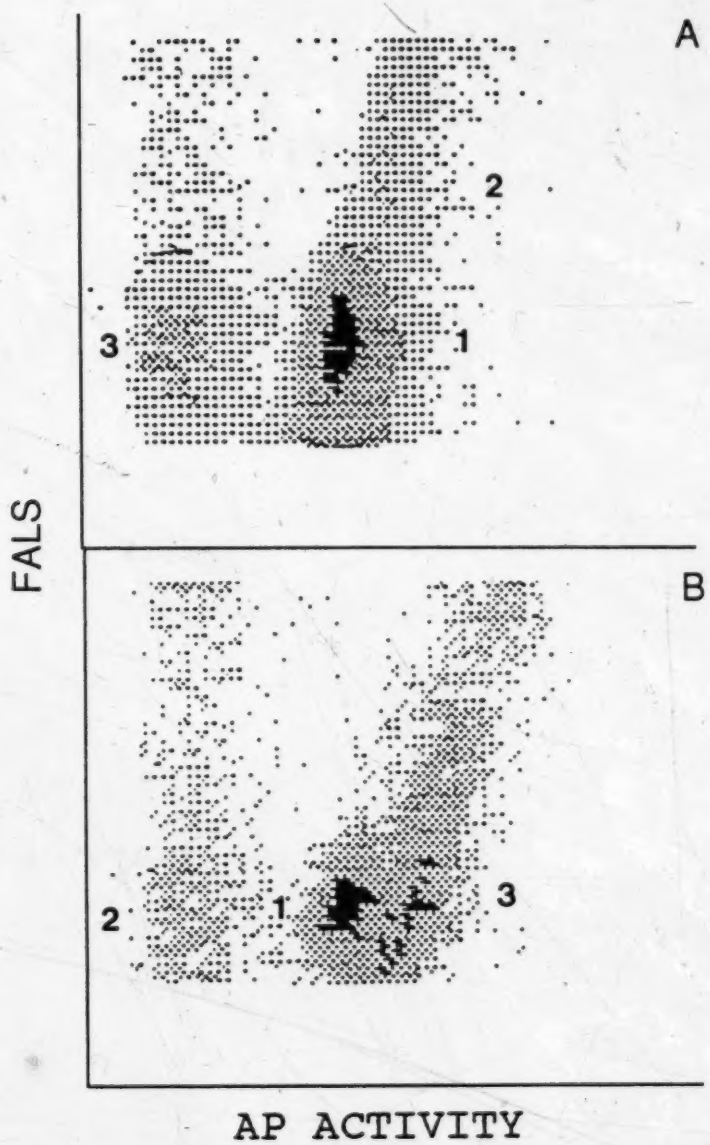

FIGURE 15. Dot plot of AP activity vs FALS of rabbit WBCs (A) and urinary cells (B).

corresponded with the two peaks produced by WBCs. A unique peak was also present, which had higher AP fluorescence than WBCs.

Two parameter dot plot of urine cell AP fluorescence distribution also revealed three populations of cells (Fig. 15, B). Population 1 and population 2 corresponded with those produced by WBCs. Population 3 represented a cell population with higher AP fluorescence than those obtained from WBCs and the same or slightly greater FALS intensity than that of neutrophils. This cell population most likely represented proximal tubular cells shed in the urine.

Relative proximal tubular cell numbers for 60 min and 45 min clamping animals were 47% and 63% respectively (Table 8).

Examination of fluorescently stained urinary samples for AP showed that the positive cells stained red (Plate 4).

3.2.C.d. ASSESSMENT USING GAMMA-GLUTAMYL-TRANSPEPTIDASE ACTIVITY

3.2.C.d.1) RABBIT WHITE BLOOD CELLS

One parameter fluorescence histogram of WBCs stained for GGT, analyzed by flow cytometry showed only one peak with relatively low GGT fluorescence (Fig. 16, A). Two parameter dot plot of FALS versus GGT fluorescence also showed only one cell population with relatively low GGT fluorescence (Fig. 17, A)

3.2.C.d.2) VOIDED URINARY CELLS

When urinary cells were analyzed for GGT fluorescence, a reproducible histogram was obtained, which had two peaks (Fig. 16, B). The peak with lower GGT fluorescence was similarly located as that of WBCs and the other peak had higher GGT fluorescence.

Two parameter dot plot of fluorescence versus FALS of urine cells stained for GGT revealed two cell populations (Fig. 17, B). Population 1 had similar FALS and GGT fluorescence intensities as those of WBCs and population 2 had higher GGT fluorescence and the same and slightly greater FALS than that of WBCs. This cell population most likely represented proximal tubular cells in the urine.

The relative proximal tubular cell number in 60 min. and 45 min. clamping animals were 66% and 67% respectively (Table 8).

Positive GGT cells were stained yellow when examined under fluorescent microscope (Plate 5).

3.2.D. CORRELATIONS BETWEEN CELL SHEDDING AND RENAL FUNCTION IN 60 MIN, 45 MIN, AND 30 MIN RENAL ARTERY CLAMPING ANIMALS

Mean Percent viability was correlated with urea nitrogen clearance in 60 min clamping model ($r=+0.7125$, $0.01<P<0.05$, Table 9).

TABLE 8

RELATIVE PROXIMAL TUBULAR CELL NUMBERS IDENTIFIED BY FLOW CYTOMETRIC AP AND GGT ANALYSES

Animal Model	Relative PTC number (%)[a]	
	AP Method	GGT Method
60 min	46.7±12.1	66.1±4.3
45 min	63.2±5.7	67.0±1.1

PTC=proximal tubular cell
a=data illustrate mean ± SE
60 min = 60 min renal artery clamping model
45 min = 45 min renal artery clamping model
30 min = 30 min renal artery clamping model

PLATE 4. Urinary cells fluorescently stained for AP activity (100X).

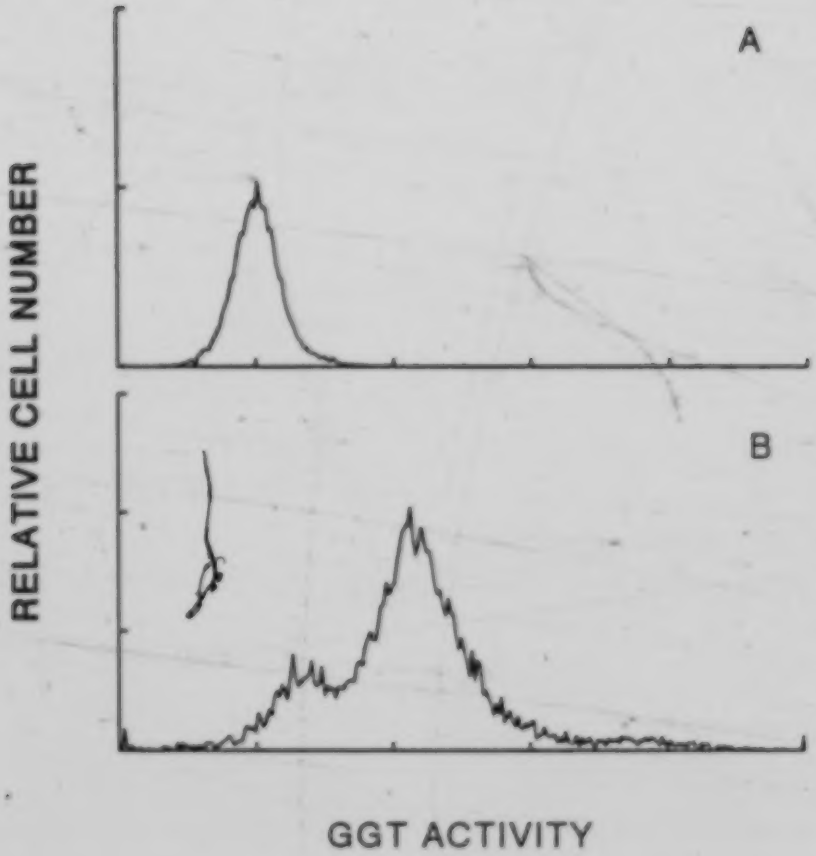

FIGURE 16. One parameter histogram of GGT activity of rabbit WBCs (A) and urinary cells (B).

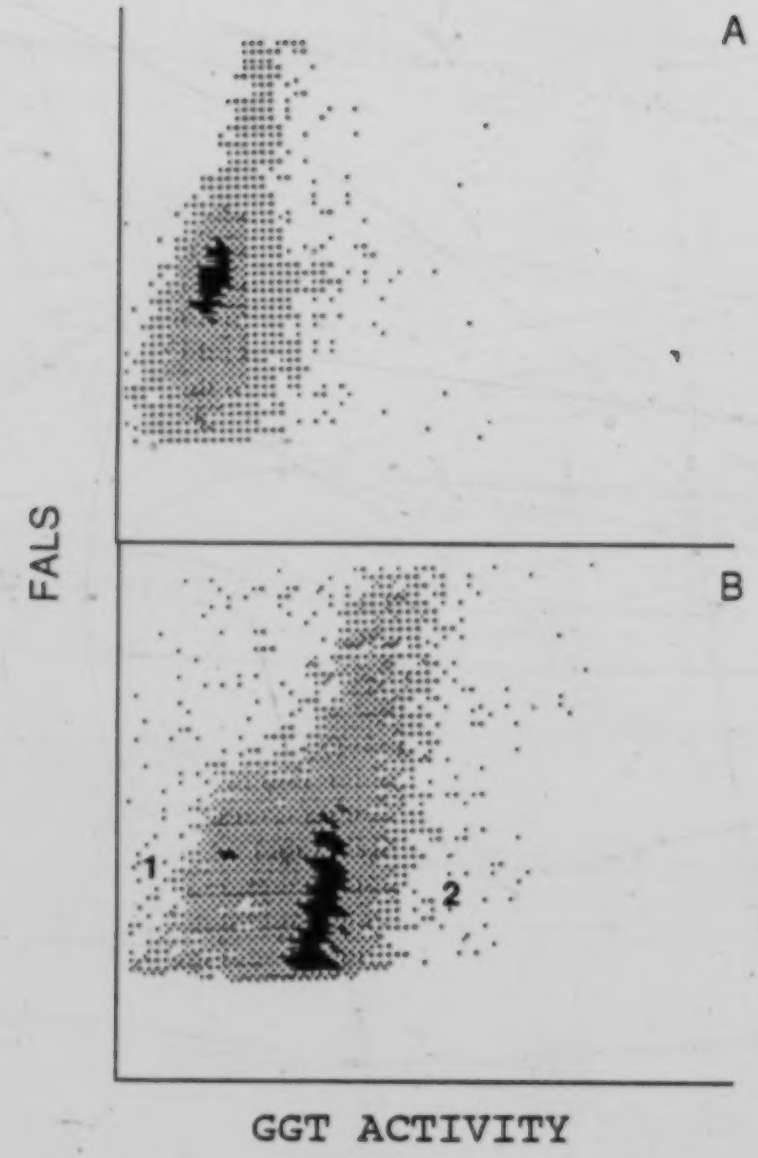

FIGURE 17. Dot plot of GGT activity vs FALS of rabbit WBCs and urinary cells.

PLATE 5. Urinary cells fluorescently stained for GGT activity.

TABLE 9

CORRELATIONS BETWEEN CELL SHEDDING AND RENAL FUNCTION IN 60 MIN, 45 MIN, AND 30 MIN RENAL ARTERY CLAMPING ANIMALS (Spearman's Rank Correlation)

1. Mean percent viability and urea nitrogen clearance in 60 min clamped animals	r=+0.7125	0.01<P<0.05
2. Total voided cell concentration and mean SUN in 60 min clamped animals	r=+0.7167	0.01<P<0.05
3. Total voided cell concentration and urea nitrogen clearance in 60 min clamped animals	r=-0.8417	0.01<P<0.05
4. Total voided cell concentration and creatinine clearance in 60 min clamped animals	r=-0.9042	0.01<P<0.05
5. Total voided live cell concentration and urea nitrogen clearance in 60 min clamped animals	r=-0.6875	0.01<P<0.05
6. Total voided live cell concentration and creatinine clearance in 60 min clamped animals	r=-0.7000	0.01<P<0.05
7. Total viable voided cells and mean urea nitrogen clearance in 45 min clamped animals	r=+0.9429	0.01<P<0.05
8. Total viable voided cells and creatinine clearance in 45 min clamped animals	r=+0.8857	0.01<P<0.05

Total voided cell concentration was positively correlated with mean serum nitrogen ($r=+0.716$, $0.01<P<0.05$, Table 9), and negatively correlated with urea nitrogen and creatinine clearance ($r=+0.8417$ and $r=-0.9042$ respectively, $0.01<P<0.05$) in 60 min clamping animals (Table 9).

Total voided live cell concentration was negatively correlated with urea nitrogen clearance and creatinine clearance ($r=-0.6875$, $r=-0.7$ respectively, $0.01<P<0.05$) in 60 min clamping model, and total viable voided cells was positively correlated with urea nitrogen and creatinine clearances ($r=+0.9429$, $r=+0.8857$ respectively, $0.01<P<0.05$) in 45 min clamping animals (Table 9).

CHAPTER FOUR

DISCUSSION

4.1. KIDNEY TUBULAR CELL EXFOLIATION IN ISCHEMIC AND TOXIC RABBIT MODELS OF ATN

The systemic study of early phase of rabbit experimental ARF of both ischemic and toxic types showed that renal tubular cell exfoliation was significantly increased. This cell shedding started early after the insult was introduced and lasted for the whole 6-h experimental period. This result is consistent with previous findings of human ATN urinary cytology studied by conventional light microscopy (Schuman and Weiss, 1981), monoclonal antibody staining method (Segasothy et al., 1988; Segasothy et al., 1989), and electron microscopy (Mandal, 1985).

No renal tubular cells were found in the urines from normal animals (urine samples collected at hour 0, before the injury was induced), suggesting that the turn over rate of normal renal epithelial cells in rabbit is low. This finding is in agreement with the newer observation of normal human renal epithelial cell exfoliation rate (Mandal, 1985), contrasting to the earlier report that renal tubular epithelial cells normally was often seen in the urine of normal individual (Rofe, 1955; Prescott, 1966).

Viable renal tubular cell shedding during ATN in patient with ATN was initially reported by Racusen et al. (1988). This study extends the observation to the early phase of rabbit models of ATN. Increased numbers of viable renal tubular cells were found in the urine of all experimental groups. This live cell shedding poses a completely new view on the kind of injury sustained by the renal tubular cells during ATN. Previous opinion on this issue was that during ATN caused either by ischemic or toxic injuries renal tubular cells undergo irreversible cell damage and necrosis in situ and cells are then detached from the surrounding tubular cells and the tubular basement membrane.

The present finding of viable tubular cell shedding suggests that cell exfoliation is a relatively early event occurring before cell death in situ. A direct consequence of this shift of concept is that it opens the possibility of preventing this live cell exfoliation, preserving the integrity of renal tubules and thus maintaining renal function in this important clinical syndrome, since cell detachment may lead to tubular backleak, tubular obstruction, and secondary renal vasoconstriction, which are important factors directly causing renal dysfunction during ATN (Levinsky et al., 1981).

Cell attachment or adhesion is believed to be mediated through three types of morphoregulatory attachment molecules (Edelman, 1989) and the cytoskeleton actin filaments (Gier, 1989). Cell junctional molecules participate in the formation

of specialized tight junctions and gap junctional complexes which in turn join cells together. In kidney, the major cell adhesion molecule is a molecule called Cadherin E (Hirano, 1987; Nagatuchi and Takeichi, 1988). It is calcium dependent and is concentrated opposite major insertion points of actin filaments. In areas other than the specialized cell membrane surface, cell adhesion molecules (CAM's) are responsible for cell to cell attachment. Substrate adhesion molecules (SAM's), of which the integrins are best characterized (Ruosahti and Pierschbacher, 1987), attach cells to the extracellular matrix or basement membrane.

During ischemic and toxic renal injury in ARF, intracellular calcium concentration may be increased. Cytoskeletal actin filaments would then be expected to contract, causing subsequent changes of cell shape and cell loosening. Decreased extracellular calcium concentration occurred in renal failure (Massry, 1974) may also cause the impaired effectiveness of cell adhesion molecules whose function is dependent of calcium influx into cells. Cell attachment mechanisms are then further compromised.

4.2. FLOW CYTOMETRIC STUDIES OF VOIDED URINARY CELLS OF RABBIT MODELS OF ARF

FDA flow cytometric analysis of urinary cells identified a cell population with a FDA fluorescence intensity lower than that of granulocytes and higher than that of RBCs. This cell population most likely represents exfoliated viable renal tubular cells. This interpretation is based on the following reasons; 1) the FALS behaviour of this cell population suggests that they are cells with a cell size similar to or larger than granulocytes, a finding consistent with the previous observations by Lindgvist and Wahlin (1975), 2) the fluorescence intensity resulted from FDA staining reflects the rate of fluorescein production through the hydrolysis of FDA by cellular nonspecific esterase and the rate of exit of fluorescein from the cell. The latter process is dependent on the integrity of the cell membrane. Exfoliated renal tubular cells are the cells in urine which undergo possible plasma membrane alterations and detach from the tubular cell membrane. Therefore it is likely that their plasma membranes are less intact compared to granulocytes, hence they have lower FDA fluorescence. Another possible explanation may be that renal tubular cells have less cellular nonspecific esterase activity than granulocytes. However there is no information in the literature concerning the relative nonspecific esterase activity of renal tubular cells versus peripheral blood cells.

Another interpretation of the result of the FDA fluorescence analysis of urinary cells may be that the higher FDA fluorescence population represents live renal tubular cells and live white blood cells and the low FDA fluorescence population represents dead renal tubular cells and dead white

blood cells. This is unlikely because to accept this conclusion one must admit that live WBCs and live renal tubular cells have the same FDA intensity and tubular cells are mostly dead since the live cell population does not seem to contain any tubular cells judged by its FALS behaviour.

Alkaline phosphatase and gamma-glutamy-transpeptidase fluorescent flow cytometric analyses of urinary cells both identified a cell population different from WBCs. These two cell populations have either higher AP activity or GGT activity than the WBCs. The most likely interpretation of these results is that they represent proximal tubular cells in the urine. This is based on the following reasons; 1) These two cell populations, like the renal tubular cells recognized on two parameter dot plot of FALS versus FDA fluorescence, have the cell size similar to or larger than that of granulocytes as shown on the two parameter histogram of blood cells. 2) Proximal tubular cells are the renal tubular cells stained most intensively for AP (Burstone, 1958) and GGT (Rutenberg et al., 1969) on a histochemically stained section of the kidney.

When the urine samples were characterized and quantified by flow cytometry, a majority of the voided cells were proximal tubular in origin (ranging from 47% to 67%). This result is in agreement with the findings of Segasothy et al. (Segasothy et al., 1988; Segasothy et al., 1989) and Lindqvist et al. (1975). However, the present study showed higher percentage of proximal tubular cells than the above mentioned observations. This discrepancy is probably due to the higher sensitivity of the flow cytometry combined with fluorescent proximal tubular marker enzyme staining.

Flow cytometry is becoming an increasingly important tool for the evaluation of urine cytology. Although it has established a strong foot-hold in oncologic DNA analysis, studies of whole cells and cell markers are still in their infancy (Landay and Bray, 1988). The current study adds to the scope of the utilization of this potentially powerful technique to the study of characteristics of whole cell in urine by applying this technique to the study of renal tubular cells shed in urine during ARF using enzymes as cell markers analyzed by fluorescent cytochemistry. The combination of computer data analysis with flow cytometry provides a simple, rapid, quantitative, and objective way to analyze urinary cells. More importantly desired cell populations, e.g. viable tubular cell, tubular cells of different nephron origin, can be separated by electronic sorting for further analysis of these cells to better understand their roles in physiological and pathological processes of the kidney.

4.3. CORRELATIONS BETWEEN RENAL TUBULAR CELL SHEDDING AND RENAL FUNCTION IN RABBIT MODELS OF ARF

In 60 min renal artery clamping model, there was a positive correlation between total voided renal tubular cells

Table 5, 1), total voided viable cells and urine output (Table 5,2). This result suggests that cell shedding is dependent upon the urine out flow or absence of obstruction in a majority of the nephron.

In $HgCl_2$-treated rabbits, percent viable cells was negatively correlated with serum creatinine concentration (Table 5, 3) and positively correlated with creatinine clearance (Table 5, 6), and in 60 min renal artery clamping animals, percent viable cells was positively correlated with clearance of urea nitrogen (Table 9, 1). These results suggest that better renal function is correlated with more live cell shedding rather than dead cell shedding. This is in agreement with the finding reported by Mandel et al. (1985) in which it was shown that the severity of urinary renal tubular cell damage was correlated with the severity of the clinical course, renal recovery, and patient survival. the exact nature of this relationship between the degree of renal tubular cell damage and renal function is uncertain at the present. It may imply that less damaged renal tubular cells in urine reflects less damage of renal tubular cells in situ, hence better renal function, whereas more dead tubular cells in urine means severe damage of renal tubular cells in situ, and the altered tubular function caused by renal tubular cell damage is responsible for the decreased renal function. On the other hand, as mentioned by Mandel, this correlation could be an indirect one, i.e. the renal tubular cells in urine undergo varying degrees of autolysis during their transit through the urinary tract or during the delayed time period before analysis of the collected urine sample. The damage occurred to these renal tubular cells would not then be a direct reflection of the degree of injury occurred to renal tubular cells in situ and the correlation is indirect. However, the fact that urine samples were collected and processed shortly after the introduction of the insult in the present study, makes it unlikely that significant autolysis has happened, and the correlation most likely represent a direct relationship between renal tubular cell damage in situ and renal function.

Two other types of correlations between cell shedding and renal function were also found. First, a positive correlation existed between total live cell shedding and clearances of creatinine and urea nitrogen in $HgCl_2$-treated animals (Table 5, 5) and 45 min renal artery clamped animals (Table 9, 7, 8), a negative correlation was found between total live cell shedding and serum creatinine concentration in $HgCl_2$-treated animals (Table 5, 3). Secondly, total cell concentration in 60 minute renal artery clamped animals is positively correlated with serum urea nitrogen (Table 9, 2) and negatively correlated with clearances of creatinine (Table 9, 4)and urea nitrogen (Table 9, 3). These correlations indicate that better renal function is associated with more cell shedding. One possible interpretation of this correlation is that more cell shedding probably reflects less tubular obstruction by

cellular component, and the latter is in turn associated with better renal function.

An alternative interpretation for the second type of correlation may be that urine output plays a role in the relationship. perhaps better renal function is associated with more urine output, hence less total cell concentration. However no statistically significant correlation was found between these two parameters. Therefore, cell number is probably related directly to renal function.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

During the initial phase of rabbit models of ATN of ischemic (renal artery clamping) and toxic (glycerol- and mercuric chloride- induced) injury, exfoliated renal epithelial cells are immediately and persistently elevated (from hour 1 to hour 6 after the introduction of the insult). This renal tubular cell shedding accompanies the reduction in renal function which also occurred early.

43% of the exfoliated renal tubular cells are still viable when assessed by trypan blue exclusion test, with the per cent of viable tubular cells being characteristic of the individual animal model. This finding of viable cell shedding may change the existing opinion about the type of cell injuries received by renal tubular cells during ATN. Cell detachment occurs before in situ irreversible cell damage and cell death. This opens the issue of preventing this live cell exfoliation and thus preserving renal function during ARF and also points to the future line of investigation of the exact process of cell exfoliation at the subcellular level.

Similar to human ATN, early phase of rabbit models of ischemic and toxic ATN is also characterized by major proximal tubular cell exfoliation when studied by fluorescent flow cytoenzymological technique. Because of the lacking of cell markers for rabbit distal tubular cells and collecting duct cells, this study was unable to provide more detailed information on the exfoliation of these two cell types.

Interesting correlations exist between renal tubular cell shedding and renal functional parameters.

Total cell and total live cell sheddings are correlated with urine output suggesting cell shedding is dependent upon urine flow.

Viability of voided renal tubular cells is correlated with renal function as assessed by serum creatinine and urea nitrogen level and clearances of creatinine and urea nitrogen, indicating that degree of urinary tubular cell damage is related with the alteration of renal function. This may suggest a relationship between renal tubular cell injury in situ and the renal function during ARF.

Another type of correlation was also found between total cell shedding, voided live cell concentration, and renal function. Better renal function was found to be related with greater number of renal tubular cells in urine. Possible interpretation of this result is that more cell shedding may indicate less tubular obstruction, which is, in turn, related with better renal function.

This study was the first one to apply flow cytometric technique to the analysis of urinary renal tubular cells. By using FDA, fluorescent alkaline phosphatase, and gamma-

glutamyl-transpeptidase staining methods, renal tubular cells and proximal tubular cells are differentiated from other cell types in urine and quantified. It was found that the majority of the voided cells were proximal tubular in origin ranging from 47% to 67%.

Systematic study of renal tubular cell shedding is a relatively new aspect in the research of ARF. The cell types and degree of damages of the urinary cell may be a good indicator of tubular cell injury in situ. Sample collecting is easy, noninvasive. Thus, further studies in this area will provide new insight in our understanding of ARF.

The present study only investigated cell shedding in the initial phase of ARF of animal models. Studies in the later time period of ARF should provide information on this cell shedding phenomenon, especially on live tubular cell shedding to determine whether it is an ongoing process, since the mechanism of fresh cell damage in the maintenance phase should be different from the initial phase.

Flow cytometry is an ideal technique to accurately differentiate and quantify different cell populations in a heterogeneous sample. It is expected that further application of this technique to urinary cell studies, such as cell sorting and identification of individual cell populations using newer cell marker, would give more detailed information about urinary cells in a pathologic situation in which reasonable number of cells can be obtained. However, a flow cytometer is a complicated instrument to operate and require certain level of expertise. One should familiarize him or herself with the machine using the more easily obtainable blood cells before attempting a formal experiment.

In conclusion, this study succeeded in characterizing renal tubular cell shedding in urine in the early phase of rabbit models of ARF of ischemic and toxic origin by LM and flowcytometry. More importantly, an association between shedding of renal tubular cells in urine and renal dysfunction was demonstrated, providing the experimental evidence for the hypothesis that renal tubular cell exfoliation may play an important role in the pathogenesis of ARF. By introducing the concept of renal tubular cell exfoliation into the preexisting picture of the pathogenesis of ARF, we may be one step closer to fully understand this important clinical syndrome.

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